# Epidemiological studies of inflammatory airway disease in horses

## Thesis

# EPIDEMIOLOGICAL STUDIES OF INFLAMMATORY AIRWAY DISEASE IN HORSES

**J. Richard Newton BVSc MSc MRCVS**

A thesis submitted for the degree of Doctor of Philosophy

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# Abstract

Studies of respiratory disease in different populations of horses were undertaken, including a case control study, using clinically apparent respiratory disease in Thoroughbred racehorses as the case definition. Controls were matched on date and training yard and data were analysed using conditional logistic regression. Disease was statistically associated with several infectious and non-infectious risk factors. Younger horses, those entering training within the last 3 months and those with *Actinobacillus/Pasteurella* spp. or *Mycoplasma felis* isolated from the trachea were at increased risk of clinical disease. When all controls with sub-clinical disease were excluded, tracheal *Streptococcus zooepidemicus* infection was significantly associated with disease.

An experimental bacterial vaccine against *S. zooepidemicus* and *Actinobacillus* spp. was evaluated for its effect on natural respiratory disease in Welsh Mountain pony foals using a blinded, randomised, controlled trial. Weekly examinations were conducted in 29 ponies, of which 12 received vaccine, 12 received placebo and 5 were untreated. Data were analysed using a multilevel modeling approach, with autoregressive variables to adjust for significant effects of disease in previous time periods. Tracheal infection with *S. zooepidemicus* was a significant risk factor for aggregated clinical scores and clinical signs of nasal discharge, cough and dyspnoea. There was evidence for a significant dose response between *S. zooepidemicus* infection and dyspnoea and inflammatory airway disease. Both pony-level and observation-level analyses demonstrated significant variation in clinical scores between ponies with transferrin haplotypes D or F2.

Tracheal and nasopharyngeal isolates of *S. zooepidemicus* from the ponies in the vaccination study were typed by polymerase chain reaction of the hypervariable region of the M-protein (5 possible types) and the 16S-23S RNA gene intergenic spacer (8 possible types). More *S. zooepidemicus* types were isolated from the trachea than the nasopharynx. There was evidence for clonal succession of types over time and novel *S. zooepidemicus* types were identified. There were apparent differences in the strength of association of different *S. zooepidemicus* types with respiratory disease in these ponies during the study period.

# Acknowledgements

I am particularly grateful to James Wood and Neil Chanter who were responsible for supervising my PhD studies. James Wood has been a constant source of advice and guidance on all aspects of these studies and his own experience of PhD study has been invaluable. Neil Chanter conducted the pony studies described in this thesis in 1996 and has provided constant encouragement and guidance with all aspects of my studies since then.

Nicola Talbot conducted much of the day-to-day microbiology from the pony study, including storage of isolates of *Streptococcus zooepidemicus.* Ross Laxton conducted all the PCR typing of *S. zooepidemicus* isolates from the pony study and passed on these skills to me so that I could conduct typing of isolates from Thoroughbreds that he cultured from samples that I submitted during 2000. Carl Robinson has also provided considerable help in understanding molecular aspects of streptococci. Ted Harding provided helpful comments on statistical and presentational aspects of earlier drafts of this thesis and Amanda Tanner and Sandra Tatum were invaluable in assisting with references. Katherine Rogers assisted with aspects of graphical presentation and Jenny Mumford has enthusiastically supported these studies throughout. I would particularly like to thank Janet Daly for her thorough and timely proof reading and helpful comments on presentation of the thesis. I am grateful to Twink Allen and his staff at the Equine Fertility Unit in Newmarket, where I conducted the latter part of my writing up.

I would also like to thank the many people who helped in the conduct of the longitudinal study of respiratory disease in racehorses in which the case control study in this thesis was nested. These include the trainers, their staff, veterinary surgeons and not least their horses. I would also acknowledge the considerable efforts of all laboratory and allied staff at the Animal Health Trust (AHT) and Mycoplasma Experience in Reigate, Surrey who processed the many samples.

I am especially grateful to the Home of Rest for Horses which funded my residency in Equine Epidemiology at the AHT and Hoechst Roussel Vet (now Intervet UK Ltd) and the Horserace Betting Levy Board who funded the studies of respiratory disease in Welsh Mountain ponies and Thoroughbred racehorses, respectively.

Last but by no means least I must acknowledge the unstinting support and tolerance provided by my dear wife Katie and adorable son James throughout the conduct and writing up of these studies and it is to them that I dedicate this thesis.

# Preface

This thesis comprises 6 sections with chapters within each section. Sections include the introduction, descriptions of a case control study of clinical respiratory disease in racehorses, a study of natural respiratory disease in Welsh Mountaion pony foals, a study of the molecular epidemiology of *Streptococcus zooepidemicus* infection in the ponies, conclusions and future work and references and appendices.

The first introduction section is a single chapter which provides an overview of equine respiratory disease and its research at the AHT and selective reviews of clinical signs of respiratory disease in the horse, the iron-binding protein transferrin and typing of the bacterium *S. zooepidemicus*.

The subsequent 3 sections describe the different studies and comprise 4 chapters each of introduction, materials and methods, results and discussion. The introduction chapters provide a brief background and aims for each study. The materials and methods chapters describe the equine populations, sampling strategies, laboratory methods and statistical approaches adopted. The results chapters summarise results of statistical analyses but with the quantity of presented data limited wherever possible and the discussion chapters discuss the relevance of these results to current knowledge of equine respiratory disease.

The penultimate section summarises conclusions for each study and discusses what future work is required from them. The final section of references and appendices includes the results of the majority of statistical analyses, which are not presented in the results chapters.

# Table of Contents

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| AHT | Animal Health Trust |
| BAL | Bronchoalveolar lavage |
| CDNS | Cough, dyspnoea, nasal discharge and submandibular lymph node |
| CF | Complement fixation |
| CI | Confidence intervals |
| CLR | Condiional logistic regression |
| COPD | Chronic obstructive pulmonary disease |
| DDSP | Dorsal displacement of the soft palate |
| EAV | Equine arteritis virus |
| EDTA | Ethylene diamine tetra-acetic acid |
| EHV | Equine herpesvirus |
| EIPH | Exercise induced pulmonary haemorrhage |
| ERV | Equine rhinovirus |
| EVA | Equine viral arteritis |
| EVU | Equine virology unit |
| HAU | Haemagglutination units |
| H&E | Haematoxylin and eosin |
| HI | Haemagglutination inhibition |
| HV | Hypervariable |
| IAD | Inflammatory airway disease |
| IGLS | Iterative generalised least squares |
| IRP | Iron regulated protein |
| IURD | Infectious upper respiratory disease |
| LRS | Likelihood ratio statistic |
| LRT | Lower respiratory tract |
| ME | Mycoplasma Experience |
| MEE | Multilocus enzyme electrophoresis |
| MELR | Mixed effect logistic regression |
| MLE | Maximum likelihood estimation |
| NFGF | Non-felis gluscose fermenting |
| NP | Nasopharyngeal |
| OR | Odds ratio |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PFGE | Pulsed-field gel electrophoresis |
| PI3 | Parainfluenza-3 virus |
| PLH | Pharyngeal lymphoid hyperplasia |
| PQL | Penalised quasilikelihood |
| RAO | Recurrent airway obstruction |
| RAPD | Random amplified polymorphic DNA analysis |
| RFLP | Restriction fragment length polymorphism |
| RIGLS | Restricted iterative generalised least squares |
| RPLN | Retropharyngeal lymph node |
| SCD | Smear cell density |
| SE | Standard error |
| SMLN | Submandibular lymph node |
| SPAOPD | Summer pasture associated obstructive pulmonary disease |
| SPF | Specific pathogen free |
| TBP | Transferrin binding protein |
| TW | Tracheal wash |
| UK | United Kingdom |
| URT | Upper respiratory tract |
| WAS | Weekly average score |

# SECTION 1

# INTRODUCTION

# CHAPTER 1

# INTRODUCTION

## 1.1 Overview

### 1.1.1 Significance of respiratory disease to equine morbidity

It has been increasingly recognised across the world that respiratory disease is an important cause of morbidity in horses and ponies. A study of the demography and diseases of the equine population of Scotland and the 5 northernmost English counties identified that respiratory disease was the second most common health problem after lameness (Mellor, 1997). The findings of the importance and impact of respiratory disease in that study was in broad agreement with those of other recent demographic studies of different types of horses in North America (Kaneene *et al.*, 1997; Gross *et al.*, 2000). In addition, studies on the causes of racehorses failing to achieve optimal training and racing performance (so-called racehorse 'wastage') conducted in the UK, Australia, New Zealand, South Africa and Germany have consistently reported respiratory disease as the second most common cause of wastage after lameness (Jeffcott *et al.*, 1982; Rossdale *et al.*, 1985; Bailey *et al.*, 1999).

Although case-specific mortality rates for most respiratory diseases are much less than for other conditions such as colic or catastrophic orthopaedic injury (Kaneene *et al.*, 1997), respiratory illness frequently clusters in both time and space, often resulting in many horses being affected simultaneously. Respiratory disease of different types occurs in horses of different ages and in individual horses is frequently chronic and recurrent in nature. Respiratory infections are more common in younger animals and can recur over many months. Chronic allergic respiratory disease is more common in older horses and frequently recurs following repeated exposure to environmental factors.

Respiratory disease, therefore, contributes to a considerable proportion of equine morbidity and in some cases, mortality. Consequently, research that contributes to a better understanding of the reasons for and ultimately ways of reducing such morbidity and mortality is ethically justified. It is with this common and fundamental aim of improving equine welfare that studies of respiratory disease in horses, over many years and in many forms, have been conducted at the Animal Health Trust (AHT) in Newmarket.

### 1.1.2 Previous equine respiratory disease research at the AHT

The AHT, as a dedicated equine research organisation, has long recognised the importance of respiratory disease in horses and the extensive and varied work conducted by its clinicians and researchers over several decades is testament to this.

Bob Cook was one of the first clinicians to report on using flexible endoscopes for the examination of the equine upper respiratory tract (Cook, 1974c). His work using endoscopy included characterising many different conditions of the upper respiratory tract (Cook, 1974c; Cook & Littlewort, 1974) including laryngeal hemiplegia, for which he was awarded a PhD in 1974 (Cook, 1974b). In addition, he identified that blood observed at the nostrils (epistaxis) in racehorses actually originated from the lungs in these animals and he postulated that this condition may have been causally associated with chronic allergic lung disease (Cook, 1974a). He also contributed to descriptions (Cook, 1976) and aetiological hypotheses (Cook & Rossdale, 1963) of this chronic allergic disease, which was subsequently termed 'chronic obstructive pulmonary disease' or COPD (Muylle & Oyaert, 1973) and has since been re-termed 'recurrent airway obstruction' or RAO (Robinson, 2001).

David Powell, an equine epidemiologist based at the AHT and supported by laboratory services provided by the Animal Virus Research Institute and the Royal Veterinary College, conducted investigations of outbreaks of suspected infectious

respiratory disease in racehorses between 1971 and 1976 (Powell, 1976; Powell *et al.*, 1978; Wood, 1999). These investigations confirmed equine influenza as an important cause of respiratory disease (Powell *et al.*, 1974b; Powell, 1975) and suggested that equine herpes virus (EHV) was associated with around half of respiratory disease outbreaks in racehorses (Powell *et al.*, 1974a; Powell, 1975; Powell *et al.*, 1978). There were also attempts made at this time to demonstrate the role of bacteria and mycoplasma in outbreaks of respiratory disease (Allam *et al.*, 1973; Allam & Lemcke, 1975) but no significant association was detected, possibly due to the inappropriate site of sampling in the upper rather than lower respiratory tract (Windsor, 1973).

Dr Jenny Mumford has been head of the equine virology unit (EVU) at the AHT since it was established in 1981 following an outbreak of paralytic EHV-1 infection on a Newmarket stud and an equine influenza epidemic that disrupted racing, both in 1979. The unit was set up to provide dedicated diagnostic services to the United Kingdom's equine industry, a role it continues to play to this day. Research in the EVU at the AHT has since contributed hugely to many aspects of equine respiratory virology, including improved knowledge of the epidemiology, immunology and pathogenesis of influenza, EHV and equine viral arteritis (EVA). This has led to improvements in management of outbreaks of viral-associated diseases and most importantly in preventive strategies, particularly through vaccination.

In addition to research on equine respiratory viruses, work at the AHT under Jenny Mumford has also re-examined the role of other organisms in different forms of respiratory disease in horses. Mike Burrell initiated this in the 1980s with the first use of flexible endoscopes to examine and sample horses' distal tracheas (Burrell, 1985; Burrell *et al.*, 1986b). Through Burrell's observations, in conjunction with detailed cytological (Whitwell & Greet, 1984) and bacteriological (Burrell *et al.*, 1986a; 1986b; Wood *et al.*, 1993a) examinations of tracheal lavages and subsequent analyses using appropriate

statistical methods (Wood *et al.*, 1993b), the distal airways have become recognised as the site of inflammatory airway disease (IAD) in young racehorses (Wood *et al.*, 1993a; Burrell *et al.*, 1994; 1996). IAD is characterised by endoscopically visible mucus and a cytologically evident neutrophilic inflammatory response (Whitwell & Greet, 1984; Burrell, 1985). A pilot longitudinal study in a Newmarket training yard suggested that IAD was not necessarily associated with viral infections but may be associated with infection with several different species of bacteria and occurred in the absence of clinical signs such as coughing and nasal discharge (Burrell *et al.*, 1994; Burrell *et al.*, 1996). Results of these studies suggested the likely pathogenic potential of *Streptococcus pneumoniae* capsule type 3 (Burrell *et al.*, 1986b), which was confirmed in subsequent experimental challenge infection in ponies (Blunden *et al.*, 1991; 1994). Analyses demonstrated a significant association between IAD and infection with *Streptococcus zooepidemicus* and/or *Actinobacillus/Pasteurella* spp. (Wood *et al.*, 1993a).

In late 1993, an outbreak of respiratory disease in a Newmarket training yard that was not attributable to recognised bacterial or viral infections, was associated with respiratory infection with *Mycoplasma felis* (Wood *et al.*, 1997a). Subsequently, a longitudinal study was conducted to specifically examine the role of bacteria, mycoplasmas and viruses in various forms of respiratory disease (nasal discharge and IAD) in racehorses (Wood, 1999). The results of the longitudinal study confirmed many of Burrell *et al's* findings; IAD was much more prevalent than upper respiratory tract disease as defined by nasal discharge and varied significantly with age, trainer and season of the year. Analyses suggested that the odds of IAD were approximately double in horses that were affected the previous month, consistent with horses, once they became affected, having IAD for an average of around 2 months. Results confirmed the association of IAD with various infections including *S. zooepidemicus, Actinobacillus/Pasteurella spp., S. pneumoniae, M. equirhinis* and EHV. Also, bacteria were much more important than

previously thought and their association with IAD was not dependent on prior viral infection.

During most of the period so far described there was very little work conducted to examine the epidemiology and aetiology of clinical presentations of respiratory disease in racehorses. There were limited reports of outbreaks of clinical upper respiratory tract (URT) disease associated with viral infections among Standardbred racehorses in Ontario, Canada (Sherman *et al.*, 1977; 1979). These studies identified that clinical disease was associated with infection with EHV-1 and equine influenza virus, particularly H7N7 subtype but not with any bacterial species isolated from the URT (Sherman *et al.*, 1977). Investigations also showed that URT disease decreased with age and outbreaks tended to recur when the proportion of the population of horses that had not previously been affected increased to 30%-40% (Sherman *et al.*, 1979).

During his time at the AHT Dr Neil Chanter contributed considerably to advances in understanding of the role of bacteria in equine respiratory disease. Much of this work was involved with infection by *Streptococcus equi* in equine 'strangles'. This work included recognition of the importance of asymptomatic carriers in strangles epidemiology (Wood *et al.*, 1993c; Newton *et al.*, 1997a; 1997b; Newton *et al.*, 1999c; Chanter *et al.*, 2000), the development of more sensitive methods of *S. equi* detection through discriminatory polymerase chain reaction (PCR) assays (Chanter *et al.*, 1997; 2000; Newton *et al.*, 2000b), development of carrier treatment regimens (Newton *et al.*, 1999c; Verheyen *et al.*, 2000) and use of modern molecular techniques in the identification and examination of specific virulence determinants (Chanter *et al.*, 1994; Chanter, 1998; Chanter *et al.*, 1999; 2000). The identification of *S. equi* virulence determinants has recently been considerably enhanced by the near completion of the sequencing of the entire genome of the strangles organism. This milestone in strangles research was made possible through a grant awarded by the Home of Rest for Horses to a collaborative

group that included Neil Chanter and should form the basis for development of the first truly efficacious strangles vaccines. In addition, Neil Chanter developed and described methods for the molecular subtyping of *S. zooepidemicus* (Chanter, 1997), techniques that have been subsequently applied to generate data that are analysed and presented in this thesis. Detailed typing of a subset of *Actinobacillus* and *Pasteurella* spp. isolated from tracheal washes demonstrated that presumptive typing tests were not sufficiently accurate in differentiating specific species of these organisms (Ward *et al.*, 1998; Wood, 1999). This finding has meant that *Actinobacillus* and *Pasteurella* spp. have been pooled for statistical purposes in the studies.

Due to the more clinical emphasis of the studies in this thesis, the remainder of this section initially reviews clinical signs of respiratory disease in horses and considers the use of 'clinical scoring' to define disease outcome. Definitions and scoring of subclinical respiratory disease are then considered briefly. There were 2 particular areas of interest in the pony study. Aspects relevant to i) equine transferrin, an iron binding protein that may act as a source of iron for respiratory bacteria and ii) subtypes of the *S. zooepidemicus* bacterium, are both considered. Finally, the design & analysis of studies of respiratory disease in horses are briefly examined.

## 1.2 Clinical signs of respiratory disease in horses

Clinical signs that are most frequently and specifically associated with equine respiratory disease are reviewed here. Association of other signs, including reduced athletic performance, swelling of the distal limbs or abnormal haematological parameters with outbreaks of respiratory disease in racehorses has been largely anecdotal (Mumford & Rossdale, 1980). However, because of their lack of specificity to disease of the respiratory tract these signs will not be considered further here.

### 1.2.1 Pyrexia

Although pyrexia (or fever) is not a sign that is specific to respiratory disease, it is frequently identified among a range of clinical signs demonstrated by horses with respiratory pathology and, therefore, warrants specific discussion.

#### *1.2.1.1 Physiology of pyrexia*

Pyrexia describes the physiological rise in body temperature above the normal range, which for non-newborn horses is between 36.5 and 38.5°C (Rose & Hodgson, 1993). This occurs because of an increase in the hypothalamic thermoregulatory set point, most commonly in response to fever-producing substances known as pyrogens, which can be either endogenous or exogenous in origin (Guyton & Hall, 1996; Eckert *et al.*, 1988; Cunningham, 2002). Pyrexia is believed to be an evolutionary physiological adaptation to reduce morbidity and mortality from infections manifested through increasing leucocyte activity (Eckert *et al.*, 1988; Cunningham, 2002).

Exogenous pyrogens, which include bacterial endotoxins such as lipopolysaccharide complexes, frequently lead to the release of endogenous pyrogenic substances from leucocytes, which act directly on the hypothalamic temperature regulatory centre (Eckert *et al.*, 1988). Endogenous pyrogens include interleukin-1 (IL-1), tumour necrosis factor (TNF), interleukin-6 (IL-6), interferon (IFN) and platelet activating factor and are involved in the acute stages of viral and bacterial diseases (Cunningham, 2002). In this mechanism of pyrexia, exogenous pyrogens appear to act indirectly by stimulation of the release of endogenous pyrogens and there is evidence in horses that successive administration of *E. coli* lipopolysaccharide endotoxin initiates tolerance (Allen *et al.*, 1996; Krumrych *et al.*, 1998).

Prostaglandins are also important components of the pathogenesis of fever and are produced by endothelial cells via the arachidonic acid cascade in response to exogenous

pyrogens and pathogenic challenge of the host. Endogenous pyrogens and prostaglandins act on a highly vacularised area of the hypothalamus known as the organa vasculosum of the lamina terminalis which is in close proximity to large numbers of thermosensitive neurons. This results in inhibition of these thermosensitive neurons by reducing their firing rate and, therefore, causes the set point of the thermoregulatory centre to rise (Guyton & Hall, 1996; Cunningham, 2002). Elevation of this set point causes the animal to conserve and produce heat by mechanisms such as shivering, peripheral vasoconstriction and piloerection until the body temperature reaches the new higher set point. This is maintained until pyrogens and prostagandins are metabolised and their production ceases, at which stage the set point decreases to normal and the animal initiates mechanisms to lose heat such as peripheral vasodilation and sweating (Guyton & Hall, 1996; Cunningham, 2002).

*1.2.1.2 Risk factors for pyrexia in respiratory disease of horses*

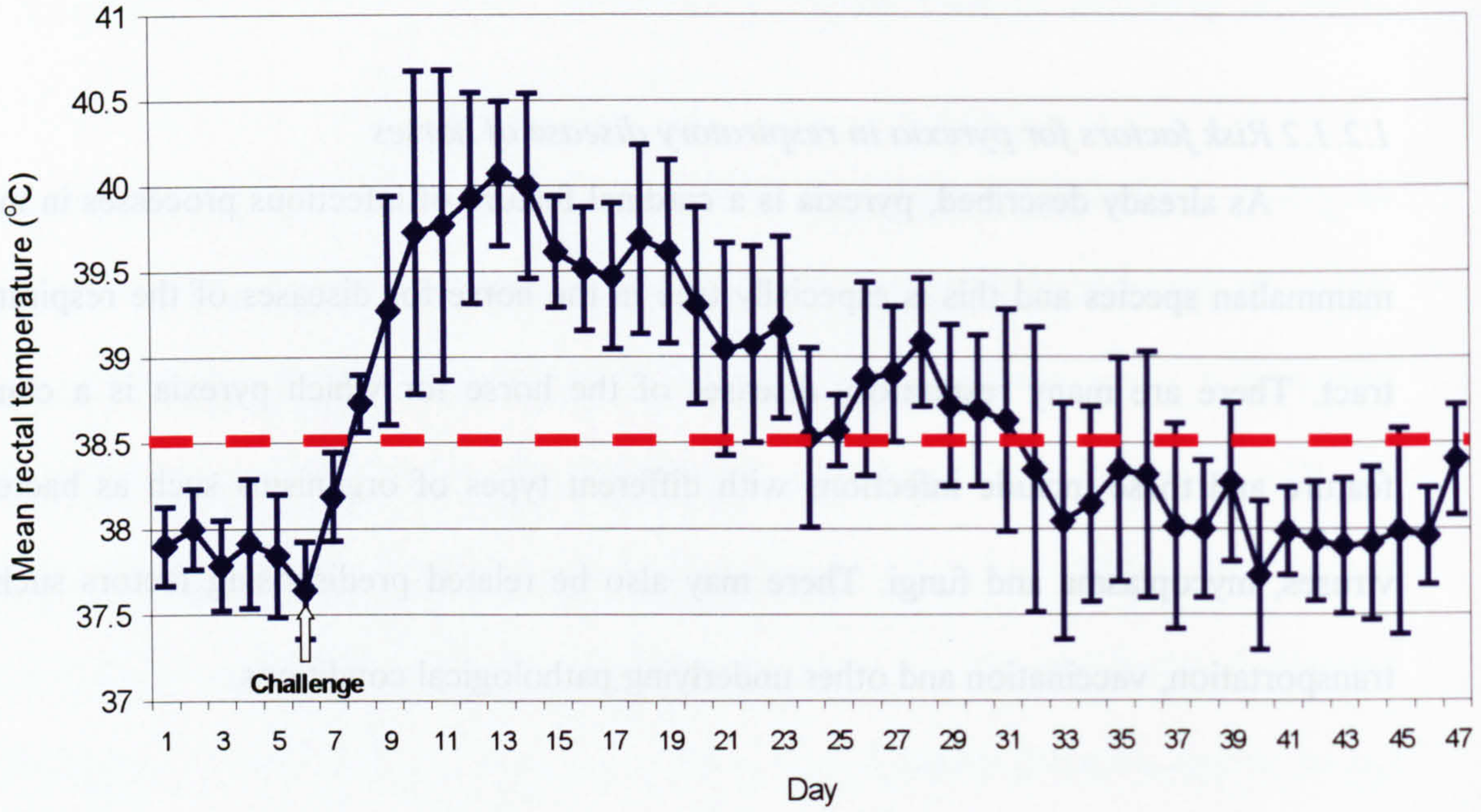
As already described, pyrexia is a cardinal feature of infectious processes in many mammalian species and this is especially true in the horse for diseases of the respiratory tract. There are many respiratory diseases of the horse for which pyrexia is a clinical feature and these include infections with different types of organisms such as bacteria, viruses, mycoplasma and fungi. There may also be related predisposing factors such as transportation, vaccination and other underlying pathological conditions.

<u>Bacterial infections</u>

Of the bacterial respiratory infections, *Streptococcus equi*, the cause of 'strangles', has been accepted as one of very few primary pathogens of the horse. Pyrexia has been reported as a clinical sign in most published outbreaks (Ebert, 1969; Piche, 1984; Timoney & Powell, 1988; Dalgleish *et al.*, 1993; Hamlen *et al.*, 1994; Fintl *et al.*, 2000;

Newton *et al.*, 2000b). In addition, previously unpublished daily clinical data from an experimental *S. equi* challenge at the AHT, following intranasal inoculation of $10^{10}$ colony forming units (cfu) of 4-hour-old *S. equi* cultures in 7 susceptible Welsh Mountain ponies, confirmed marked and prolonged pyrexia, which was initiated within a short period of challenge (Figure 1.1). These data are consistent with continued exposure of ponies to pyrogens of bacterial origin over a number of weeks following infection. The pattern of prolonged pyrexia corresponded with the persistence of *S. equi* infection identified in most of the group during the latter part of the period.

**Figure 1.1: Mean daily rectal temperatures (± 95% confidence intervals) during an experimental *S. equi* infection in 7 susceptible Welsh Mountain ponies (controls) conducted at the AHT (pyrexia [– – – –] defined as >38.5°C)**

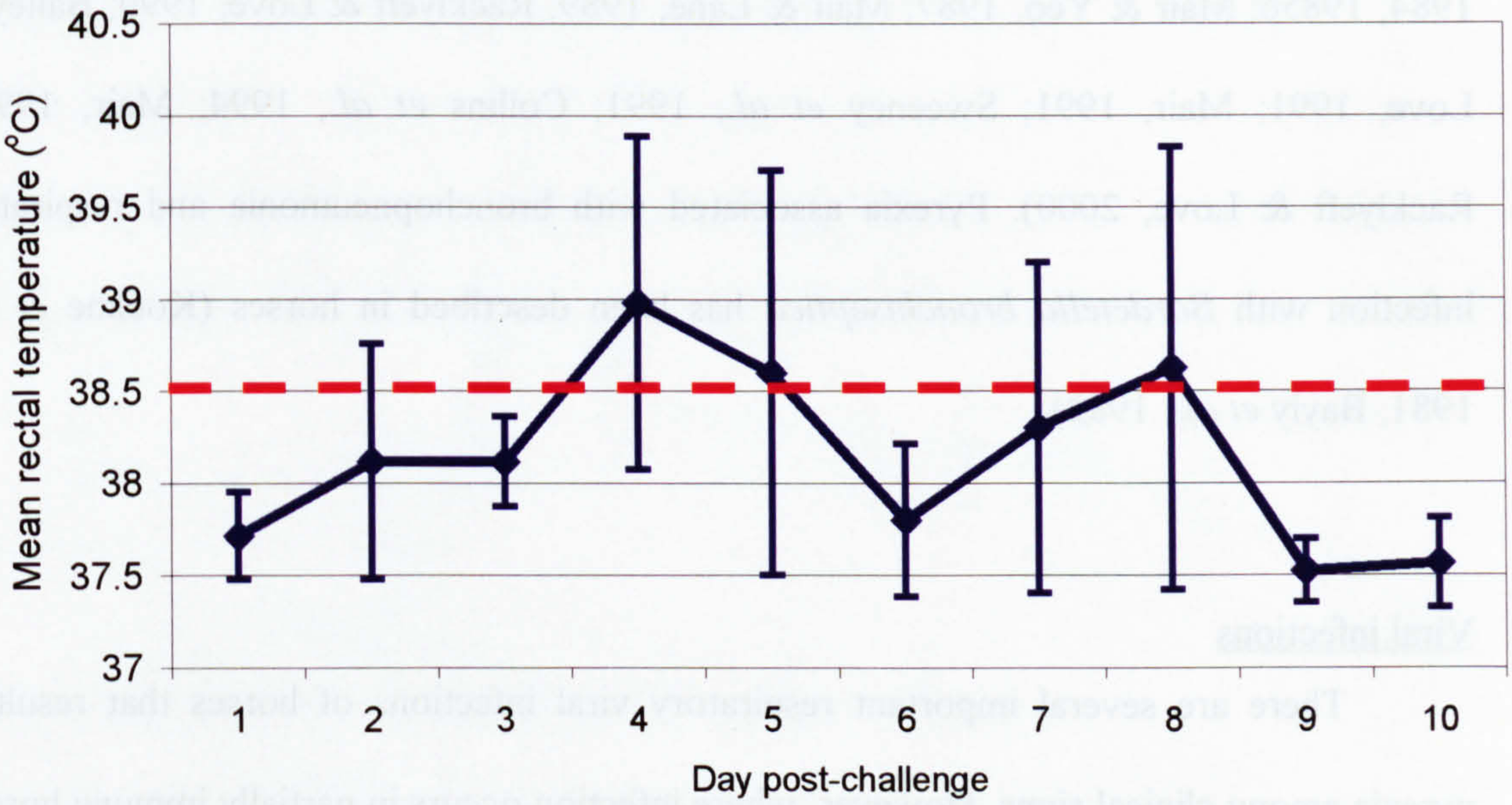



Other bacterial respiratory infections may present with signs of pyrexia but the severity of disease is highly variable.

In well-monitored young racehorses, clinical signs of respiratory disease with associated pyrexia may be extremely mild and may only be accompanied by nasal

discharge or coughing (Burrell *et al.*, 1994; 1996). In a study of pyrexia associated with respiratory signs in young Thoroughbreds, Burrell *et al.* (1994) found that the predominant organisms isolated from tracheal lavage samples were *S. zooepidemicus, S. pneumoniae* and *Pasteurella* spp., with most horses having bacterial counts $>10^4$ cfu/ml of tracheal wash. These observations corroborated results of experimental infections in horses with intravenously administered *S. zooepidemicus* (Varma *et al.*, 1984) and endoscopically intratracheal administered *S. pneumoniae* (Blunden *et al.*, 1991; 1994). Figure 1.2 summarises mean daily rectal temperature data for 7 ponies experimentally challenged with 10ml of $10^8$ cfu/ml of *S. pneumoniae* (data courtesy of A. Blunden). These data show a much reduced duration of pyrexia compared to the *S. equi* challenge infection, with a mean rectal temperature >38.5°C on only 3 of 10 days after inoculation.

**Figure 1.2: Mean daily rectal temperatures (± 95% confidence intervals) during experimental *S. pneumoniae* infections in 7 Welsh Mountain ponies conducted at the AHT (pyrexia [– – – –] defined as >38.5°C)**



In other animals, particularly foals or those being transported long distances, pyrexia may be associated with severe bacterial broncho- or pleuropneumonia, respiratory compromise and even death (Sweeney *et al.*, 1984; Bernard Strother & Mansmann, 1985; Sweeney *et al.*, 1985b; Mair & Yeo, 1987; Sweeney *et al.*, 1987a; Mair, 1989; Mair & Lane, 1989; Mair, 1991; Sweeney *et al.*, 1991; Hayakawa *et al.*, 1993; Oikawa *et al.*, 1994; Mair, 1996a; Wiegand & Schusser, 1997). *Rhodococcus equi*, although not prevalent in the UK, is recognised in foals in warm, dry climates as the cause of marked pyrexia associated with suppurative bronchopneumonia (Smith & Robinson, 1981; Zinck *et al.*, 1986; Prescott *et al.*, 1991; Hoffman *et al.*, 1993c; Knottenbelt, 1993; Burks, 1996; Kolk *et al.*, 1999). In older animals pneumonia is usually associated with mixed infections involving both aerobic and facultatively anaerobic bacterial species (Mair & Yeo, 1987; Mair & Lane, 1989; Mair, 1991; Raidal, 1995; Mair, 1996a). Bacterial species associated with equine pneumonia include *S. zooepidemicus, Pasteurella/Actinobacillus* spp., *Enterobacteria* spp., *Pseudomonas* spp., *Bacteriodes* spp., *Staphylococcus* spp. and *Bordetella bronchiseptica* (Naglic *et al.*, 1982; Raphel & Beech, 1982; Sweeney *et al.*, 1984; 1985b; Mair & Yeo, 1987; Mair & Lane, 1989; Racklyeft & Love, 1990; Bailey & Love, 1991; Mair, 1991; Sweeney *et al.*, 1991; Collins *et al.*, 1994; Mair, 1996a; Racklyeft & Love, 2000). Pyrexia associated with bronchopneumonia and respiratory infection with *Bordetella bronchiseptica* has been described in horses (Koehne *et al.*, 1981; Bayly *et al.*, 1982).

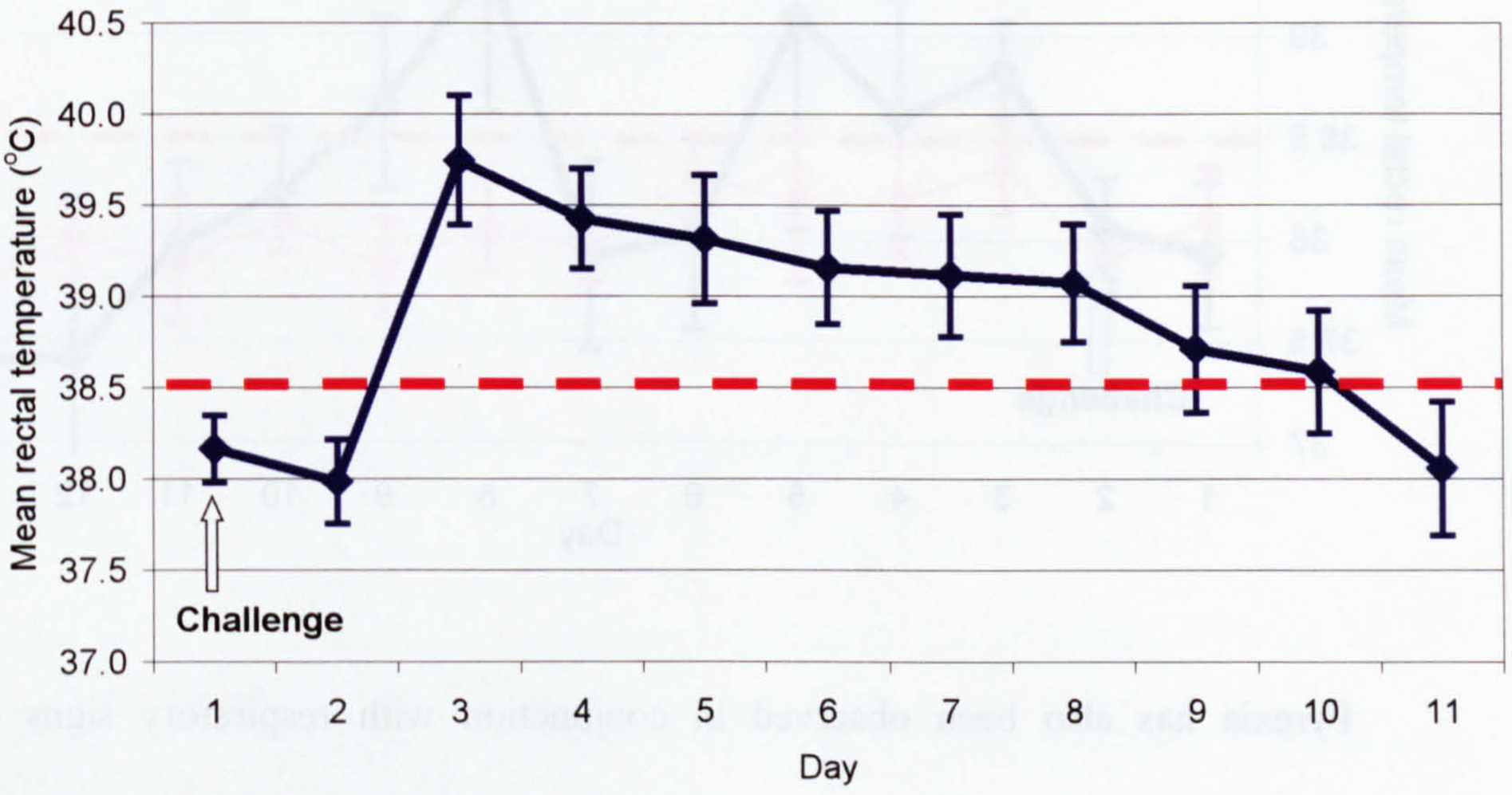
Viral infections

There are several important respiratory viral infections of horses that result in pyrexia among clinical signs. However, where infection occurs in partially immune horses, most clinical signs including fever may be significantly attenuated, although these animals

may still be infected and hence potentially infectious to other individuals (Mumford, 1998).

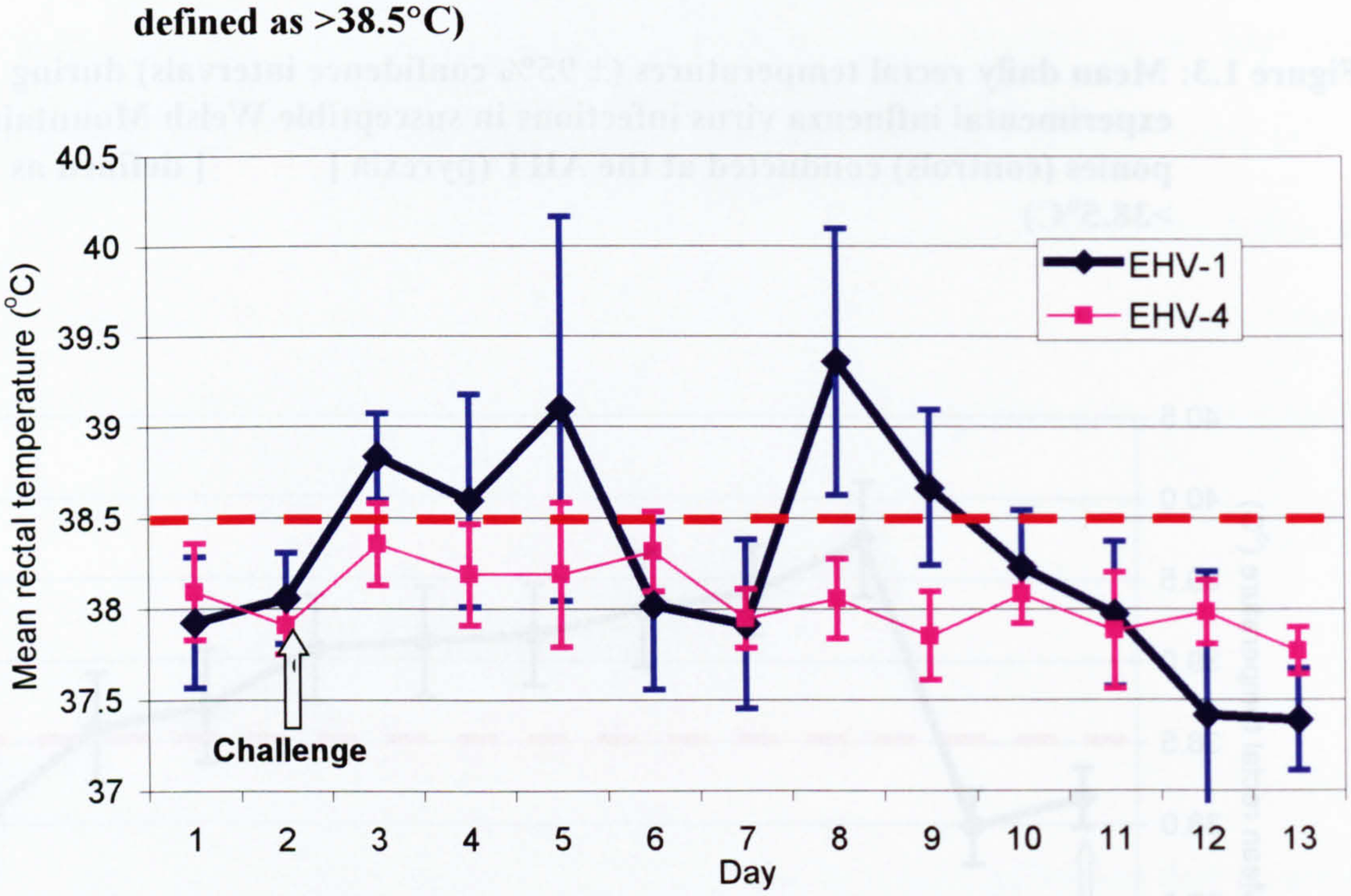
Pyrexia with other clinical signs including harsh, dry cough, nasal discharge and occasional secondary bacterial pneumonia is a feature of natural outbreaks of equine influenza virus infection in naïve horses (Gerber, 1970; Powell *et al.*, 1974b; Wood, 1991; Powell *et al.*, 1994; Newton & Mumford, 1995; Powell *et al.*, 1995; Newton *et al.*, 1999a; 2000a). Experimental infections with equine influenza viruses have allowed specific clinical signs including pyrexia to be more precisely characterised with respect to time since infection (Hannant *et al.*, 1988; Mumford *et al.*, 1990; 1994c; 1994d; Gross *et al.*, 1998; Kastner *et al.*, 1999). Figure 1.3 summarises daily rectal temperature data for susceptible control ponies experimentally challenged with various influenza viruses at the AHT (data courtesy of J. Daly and L. Spencer).

**Figure 1.3: Mean daily rectal temperatures (± 95% confidence intervals) during experimental influenza virus infections in susceptible Welsh Mountain ponies (controls) conducted at the AHT (pyrexia [– – – –] defined as >38.5°C)**



Other significant respiratory viral pathogens of horses are equine herpes viruses-1 and -4 (EHV-1 and EHV-4), both of which may have pyrexia as a characteristic clinical sign in both natural (Doll & Bryans, 1963; Allen & Bryans, 1986; Fu *et al.*, 1986; Ostlund, 1993; McCartan *et al.*, 1995; Murray *et al.*, 1998b) and experimental infections (Seahorn *et al.*, 1990; Mumford *et al.*, 1994a; Sutton *et al.*, 1998; Heldens *et al.*, 2001). Mean daily rectal temperature data from experimental infections (Figure 1.4) among susceptible Welsh Mountain ponies (controls) at the AHT have corroborated suggestions that EHV-1 infection elicits more marked clinical signs than EHV-4 (Heldens *et al.*, 2001) (data courtesy of J. Mumford).

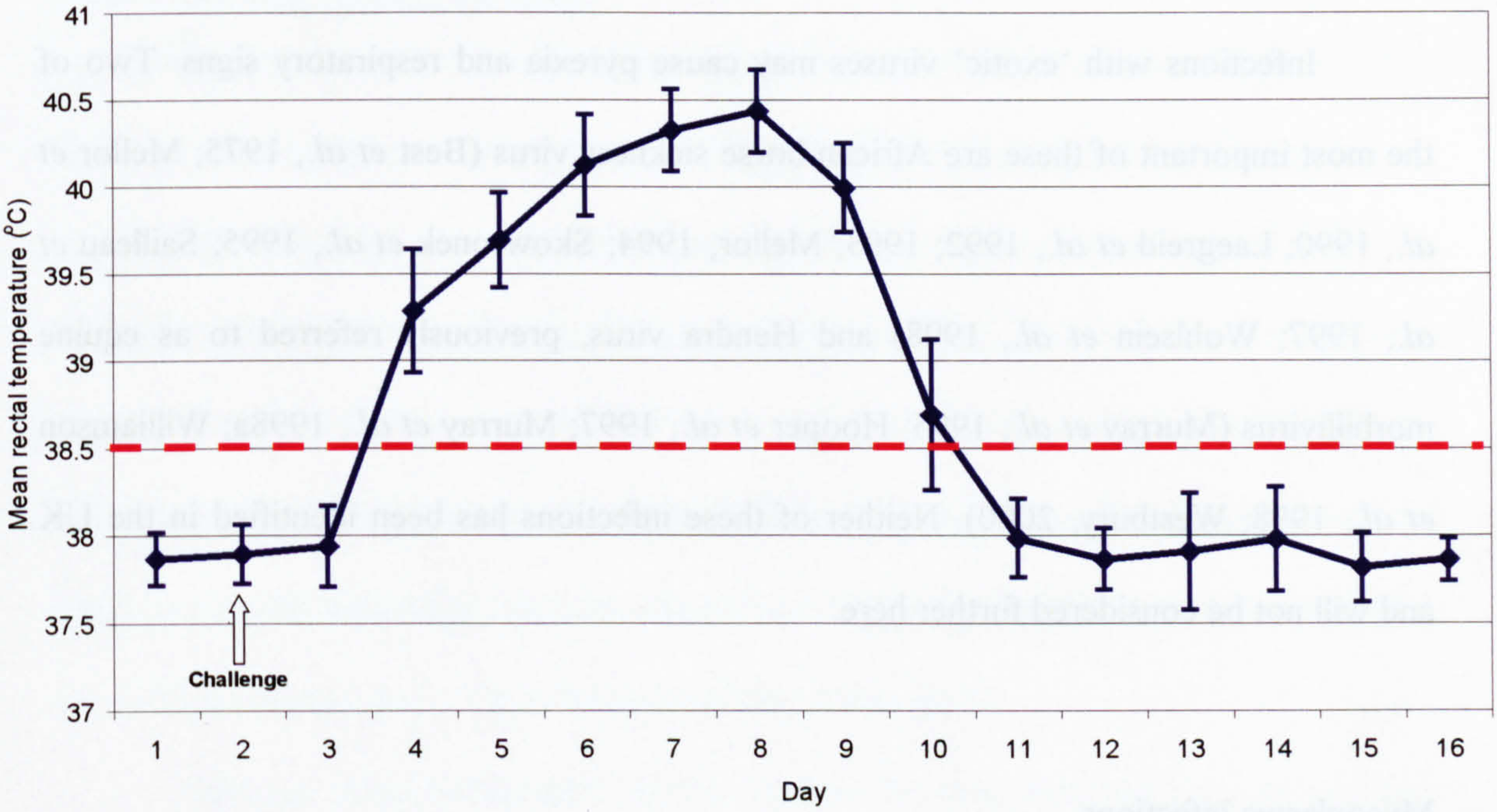
**Figure 1.4: Mean daily rectal temperatures (± 95% confidence intervals) during experimental EHV-1 and EHV-4 infections in susceptible Welsh Mountain ponies (controls) conducted at the AHT (pyrexia [– – – –] defined as >38.5°C)**




Pyrexia has also been observed in conjunction with respiratory signs during infections with equine arteritis virus in both natural (McCollum & Swerczek, 1978; Timoney & McCollum, 1990; Camm & Thursby Pelham, 1993; Monreal *et al.*, 1995;

Wood *et al.*, 1995; Del Piero *et al.*, 1997) and experimental infections (McCollum *et al.*, 1988; 1995; Paweska *et al.*, 1996; Fukunaga *et al.*, 1997; Castillo Olivares *et al.*, 2001). Figure 1.5 summarises mean daily rectal temperature data for 19 susceptible Welsh Mountain ponies (controls) experimentally challenged with equine arteritis virus at the AHT (data courtesy of J. Castillo-Olivares). These data show mean rectal temperatures >38.5°C between days 2 and 8 after infection, with mean temperatures returning to normal levels shortly before increases in virus neutralising antibody titres would usually occur, suggesting attenuation of pyrexia coinciding with immunity developing.

**Figure 1.5: Mean daily rectal temperatures (± 95% confidence intervals) during experimental EAV infections (day 0) in 19 susceptible Welsh Mountain ponies (controls) conducted at the AHT (pyrexia [– – – –] defined as >38.5°C)**




Pyrexia associated with respiratory infection by equine herpes virus-2 (EHV-2) has only been reported by one group (Palfi *et al.*, 1978; 1979) and the role of this gamma

herpesvirus in equine respiratory disease remains controversial and poorly understood (Studdert, 1996; Wood, 1999).

Although both experimental and natural infection with equine rhinovirus-1 (ERV-1) has been associated with pyrexia and other clinical respiratory signs (Plummer, 1962; Plummer & Kerry, 1962; Hofer *et al.*, 1973; Moraillon *et al.*, 1973; Powell, 1975; Studdert & Gleeson, 1978), infections with this virus are frequently subclinical (Hofer *et al.*, 1973; Studdert & Gleeson, 1978) as are infections with ERV-2 (Mumford & Thomson, 1978)

Despite an outbreak of severe upper respiratory tract disease including pyrexia occurring in a group of horses in Canada in 1961 being attributed to infection with parainfluenza-3 (PI-3) virus and from which virus was isolated (Ditchfield *et al.*, 1963), there have been few subsequent reports of equine respiratory disease associated with PI-3 infection.

Infections with 'exotic' viruses may cause pyrexia and respiratory signs. Two of the most important of these are African horse sickness virus (Best *et al.*, 1975; Mellor *et al.*, 1990; Laegreid *et al.*, 1992; 1993; Mellor, 1994; Skowronek *et al.*, 1995; Sailleau *et al.*, 1997; Wohlsein *et al.*, 1998) and Hendra virus, previously referred to as equine morbillivirus (Murray *et al.*, 1995; Hooper *et al.*, 1997; Murray *et al.*, 1998a; Williamson *et al.*, 1998; Westbury, 2000). Neither of these infections has been identified in the UK and will not be considered further here.

Mycoplasma infections

Observations of acute respiratory disease in horses including pyrexia have suggested a possible aetiological role for mycoplasma species, particularly *M. equirhinis* and *M. felis*, although concurrent infection with other organisms could not be ruled out in

these studies (Antal *et al.*, 1988; 1989; Stipkovits *et al.*, 1990; Wood & Chanter, 1996; Carman *et al.*, 1997; Wood *et al.*, 1997a).

*Mycoplasma felis* has been specifically implicated in pyrexia associated with respiratory signs in horses subsequently diagnosed with pleuritis (Ogilvie *et al.*, 1983; Rosendal *et al.*, 1986; Hoffman *et al.*, 1992a) and in one horse with pericarditis and pleuritis (Morley *et al.*, 1996). Experimental thoracic inoculation with *M. felis* in a yearling pony produced fever and respiratory signs and the organism was recovered in large numbers from pleural exudate collected 4 days after inoculation (Ogilvie *et al.*, 1983). Pyrexia was also observed in an outbreak of acute lower airway disease associated with *M. felis* infection that occurred in young racehorses in training in Newmarket in 1993 (Wood & Chanter, 1996; Wood *et al.*, 1997a).

Fungal and pneumocystis infections

Respiratory disease in response to allergic reaction to fungal and mould antigens, now referred to as recurrent airway obstruction (RAO) and previously as chronic obstructive pulmonary disease (COPD), is prevalent among older horses and is not generally characterised by pyrexia (Robinson *et al.*, 1996; Robinson, 2001). In contrast, reports of fungal infections of the airways resulting in pyrexia and respiratory signs are rare, although there may be an underestimation of its occurrence as fungal pneumonia may be frequently misdiagnosed due to its clinical similarity to bacterial pneumonia (Nappert *et al.*, 1996; Carrasco *et al.*, 1997; Sweeney & Habecker, 1999).

Although some horses with mycotic pneumonia may have had an overwhelming challenge (Ruoff, 1989; Carrasco *et al.*, 1997), others are as a result of prolonged courses of antibacterial treatment (Long & Mitchell, 1971; Pearson *et al.*, 1983; Cornick, 1990; Pace *et al.*, 1994; Nappert *et al.*, 1996). Cases may be associated with various causes of immunocompromise including use of glucocorticoids (Green *et al.*, 1987; Nappert *et al.*,

1996) pituitary adenoma (King, 1993) and myeloproliferative neoplasia (Blue *et al.*, 1987; Buechner-Maxwell *et al.*, 1994) or with presumptive dissemination from pre-existing intestinal pathology (Riley *et al.*, 1992; Johnson *et al.*, 1999).

Specific reports of airway fungal infections associated with clinical presentation of pyrexia and other respiratory signs include a case of pulmonary histoplasmosis diagnosed by radiography and cytology of transtracheal and lung aspirates (Cornick, 1990). A mare, previously treated for bacterial pneumonia, with prolonged pyrexia (up to 42°C) and ultrasonographically and radiographically apparent pneumonia also had large numbers of fungal hyphae on cytology of a transtracheal wash sample from which abundant colonies of *Scopulariopsis* spp. were also isolated (Nappert *et al.*, 1996). Fatal invasive pulmonary mycosis by mixed infection with *Aspergillus niger* and *Rhizopus stolonifer* was reported in 3 Thoroughbreds with severe signs of pneumonia and pyrexia following housing in a disused, uncleaned stable (Carrasco *et al.*, 1997). Pyrexia and tachypnoea were also observed shortly prior to death in a horse with acute pulmonary Aspergillosis following abdominal surgery (Johnson *et al.*, 1999).

Pneumonia with infection with *Pneumocystis carinii,* as with humans, occurs most commonly in animals with acquired, inherited or drug-induced immunodeficiencies (Perryman *et al.*, 1978; Ewing *et al.*, 1994). Pyrexia with respiratory signs has been reported for equine *Pneumocystis carinii* pneumonia among Arabian foals with inherited combined immunodeficiency (Shively *et al.*, 1973; Perryman *et al.*, 1978), in a Quarter horse foal (Ewing *et al.*, 1994) and Throughbred foals without evidence of immunosuppression (Whitwell, 1992; Peters *et al.*, 1994; Tanaka *et al.*, 1994).

Other factors

Various factors associated with transportation may be important in predisposing to respiratory disease with pyrexia in horses, a phenomenon commonly referred to as

'shipping fever' (Mair & Lane, 1989; Leadon *et al.*, 1991; Hayakawa *et al.*, 1993; Oikawa *et al.*, 1994; Leadon, 1995; Oikawa *et al.*, 1995). In addition to factors such as increased stress and associated immunosuppression and increased exposure to environmental irritants, an important factor is confinement of horses with their heads in an upright position. Maintaining horses with their heads in an upright position results in accumulation of airway secretions in the lower respiratory tract and consequently increases bacterial numbers by interfering with tracheal mucociliary transportation (Racklyeft *et al.*, 1991; Raidal *et al.*, 1995; 1996; 1997a; 1997c).

There have been occasional reports of pyrexia and respiratory signs in horses shortly following vaccination, including with vaccines against equine influenza (Dixon *et al.*, 1996) and *S. equi* (Smith, 1994). However, such adverse reactions are less commonly reported with modern vaccines and when they occur they are frequently attributable to concurrent infection with another organism, such as EHV (Dixon *et al.*, 1996) (AHT unpublished observations).

There are a number of reports of pyrexia associated with respiratory signs in horses which are attributed to very unusual conditions and these include pleuropulmonary abscessation secondary to a gastric foreign body (Tremaine *et al.*, 1995), pleuropneumonia associated with pulmonary hydatidosis (McGorum *et al.*, 1994), mitral regurgitation and congestive heart failure (Reef *et al.*, 1998) and iatragenic lipoid pneumonia secondary to inappropriate nasogastric deposition of mineral oil (Scarratt *et al.*, 1998).

### 1.2.2 Nasal discharge

The nares (or nostrils) are the most rostral anatomical site of the respiratory tract and so, by definition, signs of abnormal discharges that are observed at this location are usually indicative of some form of respiratory pathology. As the nares are anatomically

linked via the nasal passages and pharynx to the oesophagus and gastrointestinal tract, it is possible that abnormal nasal discharge may arise from a dysfunction of swallowing (dysphagia), oesophageal obstruction ('choke') or gastric reflux (Rose & Hodgson, 1993). As these conditions are usually easily distinguished from disease of the respiratory tract by the presence of food material in the discharge and other non-respiratory clinical signs, they will not be considered further here.

It is important that the anatomy of the entire respiratory tract is considered when reviewing possible causes of nasal discharge as a clinical sign of respiratory disease in horses. Causes of nasal discharge relating to specific anatomical sites (i.e. the nares and nasal passages, paranasal sinuses, pharynx and guttural pouches and larynx) in the upper respiratory tract (URT) are discussed, but were not the specific focus of study for this thesis. Diseases producing nasal discharges originating from the trachea and distal airways include EIPH and excessive airway mucus.

### *1.2.2.1 Nasal discharges originating from diseases of the upper respiratory tract*

Nares and nasal passages

The equine nasal passages are linked to the exterior by the nares, which are capable of expansion during exercise to maximise air intake and are where nasal discharges are observed (Lakritz *et al.*, 1997).

The bilateral nasal passages are divided medially by the median septum with the space divided into 3 nasal meati (spaces) by the ventral and nasal conchae, which are highly vascularised, scrolled turbinate bones that contribute to regulating airflow and air temperature and removal of large-sized particles by generating turbulence over their large surface area. The ventral meatus is larger than either the middle or dorsal meati and is usually the only route by which endoscopes, nasopharyngeal swabs and nasogastric tubes are able to pass during veterinary investigation or treatment.

Nasal discharges may occasionally be associated with pathology and infection of the nasal passages, usually presenting as abnormal respiratory noise or dyspnoea due to impaired airflow from space occupying lesions within the nose (Leyland & Baker, 1975; Reed *et al.*, 1979; Roberts *et al.*, 1981; Hodgin *et al.*, 1984; Shaw *et al.*, 1987; Nappert *et al.*, 1988; Casper *et al.*, 1994; Richardson *et al.*, 1994b). Lesions identified in such cases have included nasal amyloidosis (Shaw *et al.*, 1987; Nappert *et al.*, 1988; Casper *et al.*, 1994), coccidiomycosis and cryptococcal granuloma (Reed *et al.*, 1979; Roberts *et al.*, 1981; Hodgin *et al.*, 1984) and various other neoplastic and granulomatous lesions (Leyland & Baker, 1975; Richardson *et al.*, 1994b). Unilateral congenital choanal atresia, characterised by obstructing tissue between a nasal passage and the nasopharynx, has been a rare cause of unilateral mucopurulent nasal discharge in young foals, with the discharge appearing contralateral to the atresia (Hogan *et al.*, 1995).

In addition to the dorsal and ventral conchae, the ethmoturbinate bones also occupy space in the dorsocaudal region of the nasal passages and are the location for formation of progressive ethmoid haematomas, which are frequently implicated in serosanguinous nasal discharges or epistaxis (Cook & Littlewort, 1974; Leyland & Baker, 1975; Specht *et al.*, 1990; Laing & Hutchins, 1992; Nickels, 1993; Behring, 1999; Tremaine & Dixon, 2001). Foreign bodies located in various different sites in the respiratory tract and associated trauma and infection may be the source of purulent nasal discharges in horses (Arvidsson, 1981; Urquhart *et al.*, 1981; Brown & Collier, 1983; Duckett *et al.*, 1983; Mair, 1987; Scheidemann *et al.*, 1993).

Paranasal sinuses

In the horse there are 6 paired paranasal sinuses (dorsal conchal, middle conchal, ventral conchal, maxillary, frontal and sphenopalatine), which as diverticula of the nasal cavity each connect directly or indirectly to it. The maxillary sinus is the largest paranasal

sinus and contains the roots of the upper 3rd, 4th, 5th and 6th cheek teeth. Consequently, the volume of the maxillary sinus effectively increases with age as the cheek teeth grow out to compensate as they are worn down.

Discharges draining from the paranasal sinuses into the nasal passages may be swallowed or appear at the nostrils as nasal discharges. Conditions causing this include sinus cysts, primary bacterial and mycotic sinusitis, neoplasia, and secondary sinusitis, most commonly due to tooth root abnormalities in the maxillary sinus (Leyland & Baker, 1975; Cannon *et al.*, 1976; Moor *et al.*, 1982; Boulton, 1985; Chan & Collins, 1985; Coumbe *et al.*, 1987; Lane, 1987; Lane *et al.*, 1987a; 1987b; Schumacher *et al.*, 1987; Hilbert *et al.*, 1988; Beard *et al.*, 1990; Sundararaj & Viswanathan, 1992; Latimer & Thrall, 1994; Henninger & Reifinger, 1998; Dixon & Head, 1999; Head & Dixon, 1999; Dixon *et al.*, 2000; Feige *et al.*, 2000; Tremaine & Dixon, 2001).

Pharynx and guttural pouches

The pharynx anatomically constitutes the junction between the respiratory and gastrointestinal tracts, with the soft palate within the pharynx comprising both the ventral border of the respiratory tract (nasopharynx) and the dorsal border of the gastrointestinal tract (oropharynx). The pharyngeal openings of the guttural pouches, large air-filled diverticula of the equine Eustachian (auditory) tubes, are situated in the lateral walls of the nasopharynx.

The exact role of the guttural pouches is not clear, although recent studies have provided evidence that they are part of an important mechanism for the cooling of blood entering the brain during strenuous exercise (Baptiste, 1998).

When the horse lowers its head to the ground the pharyngeal openings are the most dependent (ventral) part of the guttural pouches and this facilitates natural drainage of any accumulated fluid from the pouches into the pharynx, where they are either

swallowed or appear as a nasal discharge. The dorsal nasopharynx comprises the ventral floor of the guttural pouches with associated lymphoid tissue and a caudal dilation of the pharynx known as the pharyngeal recess. The retropharyngeal lymph nodes (RPLN) are situated bilaterally and slightly more caudally than the pharynx and are also in close apposition with the ventral aspect of the guttural pouches. The RPLNs receive lymphatic drainage from a large part of the caudal head including the tongue, palate, tonsils, caudal nasal cavity and sinuses, guttural pouches, larynx, pharynx and the more rostral parotid and submandibular lymph nodes.

The respiratory tract continues beyond the pharynx through the ostium of the larynx. Various cartilages of the larynx protrude into the caudal pharynx, supported by the hyoid apparatus, of which the stylohyoid bone transects each guttural pouch. The junction between the larynx and pharynx is bounded by bilateral continuations of the soft palate known as the palatopharyngeal arches and the reflection of the mucosae onto the laryngeal cartilages as they protrude is the pyriform recess.

Strangles, caused by infection with *S. equi*, is the most common cause of nasal discharge with its origin in the pharynx and guttural pouches (Breuer *et al.*, 1975; Knight *et al.*, 1975; Rooney, 1979; Nyack *et al.*, 1981; 1983; Noren, 1985; Sweeney *et al.*, 1987b; Wood *et al.*, 1993c; Bentz *et al.*, 1996; Adkins *et al.*, 1997; Newton *et al.*, 1997b; Fintl *et al.*, 2000; Verheyen *et al.*, 2000). Strangles in horses frequently results in both pharyngitis and migration of *S. equi* to the RPLNs with subsequent abscessation (Knight *et al.*, 1975; Rooney, 1979; Nyack *et al.*, 1983; Wood *et al.*, 1993c; Newton *et al.*, 1997b; Judy *et al.*, 1999; Fintl *et al.*, 2000). The close apposition of the medial RPLNs to the guttural pouches means that rupture of these lymph nodes results in guttural pouch empyema that is frequently shortly followed by the appearance of a profuse mucopurulent nasal discharge, particularly when the horse lowers its head to the ground.

In a series of 46 cases of RPLN infections reported in Australia, there was a history of contact with *S. equi* infection in the preceeding 8 weeks in 39% of cases and 20% of all cases presented with a purulent nasal discharge (Golland *et al.*, 1995).

Other infections of the guttural pouches that have resulted in nasal discharges are less common but include *Pseudomonas aeruginosa* (Govan *et al.*, 1992) and *Pasteurella multocida* (Jaeschke & Weiler, 1998). The majority of descriptions of guttural pouch mycosis report that cases present with blood appearing at the nostrils (epistaxis) rather than purulent discharges and/or dysphagia (Breuer *et al.*, 1975; Mongeon, 1977; Nation, 1978; Owen & McKelvey, 1979; Grabner, 1984; Church *et al.*, 1986; Ryan *et al.*, 1992; Junot *et al.*, 1999). This is presumably because the mycosis remains clinically inapparent prior to either the rupture of large blood vessels, which results in acute haemorrhage or dysphagia due to disruption of cranial nerves VII, IX, X and XI which traverse the dorsal aspect of the guttural pouches. However, one study from Japan described 3 cases of mycosis in which respiratory signs including purulent nasal discharges had been observed for about one month before haemorrhage occurred (Takatori *et al.*, 1984). The specific aetiology of the mucopurulent nasal discharges was not determined in these cases. Another report from the UK described a horse with bilateral nasal discharge of 2 month's duration prior to epistaxis and isolation of *Aspergillus fumigatus* from a necrotic guttural pouch lesion at *post mortem* examination (Dixon & Rowlands, 1981).

Both congenital and acquired defects of the pharyngeal region in horses may result in nasal discharge in conjunction with other respiratory signs including inhalation pneumonia. The most commonly reported congenital defect in this region is cleft palate, which usually results in the discharge of food or milk from the nostrils (Haynes & Qualls, 1981; Shappell *et al.*, 1989; Dixon *et al.*, 1993a; Giles *et al.*, 1993; Puyalto Moussu *et al.*, 1998). Hypoplasia of the soft palate has been reported in a foal with bilateral nasal discharge, dyspnoea and aspiration pneumonia (Riley *et al.*, 1991) and a 4-year-old mare

with a chronic bilateral nasal discharge (Proudman *et al.*, 1991). Cases of rostral displacement of the palatopharyngeal arch may present with nasal discharges containing food material (Deegen & Klein, 1987; Dixon *et al.*, 1993a). Subepiglottic cysts have been found in foals with bilateral nasal discharges, coughing and aspiration pneumonia (Stick & Boles, 1980; Sheridan, 1990).

Larynx

The larynx forms the junction of the respiratory tract between the pharynx and the trachea and comprises a series of conjoined cartilages, which include single cricoid, thyroid and epiglottic cartilages and paired arytenoid, corniculate and cuneiform cartilages. The larynx is supported from the base of the skull by the hyoid apparatus.

Through its range of normal movement, facilitated by the hyoid apparatus and attachments to the base of the tongue and soft palate, the larynx functions to prevent air from entering the oesophagus and digestive system as well as stopping food and saliva from entering the trachea and respiratory tract. Normal laryngeal and pharyngeal function is a complex process that is controlled by synchronisation of multiple muscles innervated by branches of the IXth, Xth and XIth cranial nerves.

Laryngeal malfunction most commonly occurs in larger horses from idiopathic, ipsilateral dysfunction of the recurrent laryngeal nerve branch of the Xth cranial (vagus) nerve, resulting in left-sided laryngeal hemiplegia. This condition leads to obstruction of the laryngeal opening (rima glottis) during maximal exertion due to inspiratory dynamic collapse of the left arytenoid cartilage across the rima glottis. Restricted abduction of the left arytenoid cartilage is usually confirmed either endoscopically or by laryngeal palpation in athletic horses that have been presented for characteristic respiratory noise and associated poor performance. The condition may be corrected surgically by a range of different procedures, including partial arytenoidectomy (Speirs, 1986; Hay *et al.*, 1993),

ventriculectomy or 'Hobday' and prosthetic laryngoplasty or 'tieback' (Rose & Hodgson, 1993).

It is unusual for the primary condition of laryngeal hemiplegia to present with a nasal discharge where as it is not uncommon that horses develop discharges either as a temporary sign shortly after surgery or more rarely as a long-term complication of surgical correction of the condition (Hawkins *et al.*, 1997; Speirs, 1986).

*1.2.2.2 Nasal discharges originating from diseases of the trachea and distal airways*

The trachea, due to the series of incomplete concentric cartilage rings running along its length, is effectively a semi-rigid tube that extends from the larynx to its bifurcation into the primary cartilaginous bronchi at the carina (Lakritz *et al.*, 1997). Thereafter, there is further multiple branching of the conducting airways through several generations of bronchi to non-cartilagenous, smooth muscle-walled bronchioles and then terminating in the alveolar ducts and acini, where gas exchange occurs with the pulmonary capillary blood supply (Lakritz *et al.*, 1997; Pirie *et al.*, 1990).

The airways are lined by fluid which between the trachea and bronchioles is a bilayer comprising a low-viscosity, periciliary basal layer and an overlying mucus layer (Dixon, 1992; Robinson, 1997c). This bilayer protects the airways in a number of different ways including trapping of inhaled material, absorption of inhaled chemicals and gases, humidifying inspired air, maintaining hydration of the mucous membrane and contributing to immunological defence of the airway by providing a medium for inflammatory cells, immunoglobulins, lactoferrin and lysozyme (Dixon, 1992). Material trapped in the fluid lining the airways is removed proximally through movement of the mucus by the co-ordinated beating motion of the cilia on the epithelial surface, the so-called 'mucociliary escalator' (Dixon, 1992; Robinson, 1997c). The term 'mucopus' is often used interchangeably with mucus and describes respiratory secretions that have a purulent

appearance characterised by a yellow or green discolouration (Dixon, 1992; Robinson, 1997c). The discolouration of mucopus may arise with increased amounts of neutrophilic or bacterial DNA (Robinson, 1997c), with myeloperoxidase, released from leucocytes in static secretions (Dixon, 1992), or more rarely with high eosinophil levels (Dixon, 1992). The gross visible differentiation of mucus and mucopus is subjective and for the purposes of this thesis no distinction will be made between the 2 terms.

Conditions that result in excessive material being cleared from the distal airways to the pharynx may result in the appearance of abnormal nasal discharges. As well as the effects of the underlying pathological conditions, additional factors that may be important in contributing to the appearance of nasal discharge include i) the position of the horse's head and neck (i.e. lowered versus raised) ii) the action of mucokinetic drugs (i.e. those effecting mucociliary clearance) such as mucolytics, bronchodilators and bronchomucotropic agents (Dixon, 1992; Robinson, 1997b), iii) exercise and iv) coughing. Exercise has been found to enhance the clearance of material from the lower airways into the trachea (Burrell, 1985; Lumsden *et al.*, 1995) and in recent studies of IAD in racehorses endoscopy has usually been conducted following exercise (Wood, 1999; Chapman *et al.*, 2000).

Exercise induced pulmonary haemorrhage (EIPH)

Blood appearing at the nostrils or epistaxis, as already discussed, may originate from URT pathology such as ethmoid haematomas (Cook & Littlewort, 1974; Leyland & Baker, 1975; Specht *et al.*, 1990; Laing & Hutchins, 1992; Nickels, 1993; Behring, 1999; Tremaine & Dixon, 2001) or guttural pouch mycoses (Breuer *et al.*, 1975; Mongeon, 1977; Nation, 1978; Owen & McKelvey, 1979; Dixon & Rowlands, 1981; Grabner, 1984; Church *et al.*, 1986; Ryan *et al.*, 1992; Junot *et al.*, 1999).

Epistaxis occurring during or soon after exercise has been recognised in horses for centuries (Pascoe & Wheat, 1980; Pascoe & Raphel, 1982) but it was only with endoscopic examination of the equine trachea and lower respiratory tract that this haemorrhage was confirmed to originate from the lungs (Cook, 1974). Lower airway endoscopy also demonstrated that lung bleeds in exercised horses were actually far more prevalent than epistaxis (Pascoe & Wheat, 1980, Pascoe & Raphel, 1982) and the term 'exercise induced pulmonary haemorrhage' or EIPH, was adopted for this condition (Pascoe *et al.*, 1981). Since then there has been much research and scientific debate directed towards determining the definitive cause and pathogenesis of EIPH (and hence epistaxis) in horses.

Cook (1974a), in his review of epistaxis in the racehorse, attributed the condition to underlying respiratory disease, particularly recurrent airway obstruction (RAO), but no data from non-affected control horses were presented to strengthen his argument. Small airway obstruction as a cause of EIPH was proposed and justified in detailed physiological terms (Robinson & Derksen, 1980) and EIPH was later reproduced in horses with experimentally induced allergic lung disease (Derksen *et al.*, 1992). Clarke (1985) reviewed existing knowledge of EIPH and proposed that the primary mechanism was mechanical stress in the dorsocaudal region of the lung, the predominant site of pulmonary pathology (O'Callaghan *et al.*, 1987). It has been hypothesised that upper airway obstruction was the cause of EIPH (Cook *et al.*, 1988) and that stress failure of pulmonary capillaries was the specific mechanism for the condition (West *et al.*, 1993). More recently possible roles of oxidant injury in the pathogenesis through nitrous oxide production (Mills & Higgins, 1997) and locomotory impact induced trauma as the underlying cause of EIPH have been proposed (Schroter *et al.*, 1998).

However, failure to completely reconcile EIPH to any one of these many and diverse theories suggests that the aetiology and pathogenesis of EIPH is actually a

complex interplay of many factors relating to both the physiology and pathology of pulmonary function and exercise. Although the association of EIPH with exercise intensity has been well documented (Raphel & Soma, 1982; Sweeney & Soma, 1983; Burrell, 1985) and most (but not all) observational studies have shown an increased prevalence with increasing age (Cook, 1974; Raphel & Soma, 1982; Clarke, 1985; Chapman *et al.*, 2000), the epidemiology of EIPH and epistaxis has been poorly described.

A recent study of risk factors associated with EIPH-related epistaxis in racing horses in Japan showed that blood at the nostrils was more prevalent in older horses, in those racing distances ≤1600 metres, in females and in steeplechasers compared to flat horses (Takahashi *et al.*, 2001). Recent analyses of data from a longitudinal study of respiratory disease (Wood, 1999) have provided evidence that EIPH in young Thoroughbreds during training was associated with increasing age, different seasons of the year, airway inflammation (and mucus) and evidence of fungal material in the airways (Newton & Wood, 2002).

Excessive airway mucus

As with EIPH and epistaxis, excessive mucus being cleared from the lungs by mucociliary clearance may result in the appearance of material as a mucoid (or mucopurulent) nasal discharge. In common with the relationship between blood in the airways and epistaxis, excessive mucus in the respiratory tract is much more prevalent than abnormal, mucoid or mucopurulent nasal discharge (Wood *et al.*, 1998; Wood, 1999).

There has been considerable confusion, probably due to unquestionable clinical and cytological similarities, in the classification of the condition of excessive mucus in the airways of horses of different ages. However, it now appears generally accepted that there

are 2 epidemiologically distinct equine respiratory disease syndromes that manifest as an airway neutrophilic inflammatory response characterised by excessive airway mucus.

Inflammatory airway disease (IAD), which has been most intensively studied in racehorses (Sweeney *et al.*, 1992; Burrell *et al.*, 1996; Moore, 1996; Wood *et al.*, 1998; Wood, 1999), occurs in young horses and *decreases* in prevalence with increasing age (Burrell *et al.*, 1996; Wood *et al.*, 1998; Wood, 1999). In contrast, recurrent airway obstruction (RAO), previously called chronic obstructive pulmonary disease (COPD) or 'heaves', occurs in older animals (usually at least 7-years-old), is thought to be allergic in origin and *increases* in prevalence with increasing age (Cook, 1976; McPherson *et al.*, 1979a; Derksen, 1993; Dixon *et al.*, 1995b; Robinson, 2001). The syndrome 'summer-pasture associated obstructive pulmonary disease' (SPAOPD), which is clinically and pathologically identical to RAO and as the name suggests occurs in older horses at pasture, may also be associated with nasal discharge (Seahorn & Beadle, 1993; Seahorn & Beadle, 1994; Mair, 1996b; Seahorn *et al.*, 1996; Hudson, 1999; McGorum & Dixon, 1999; Costa *et al.*, 2000).

These disease syndromes may or may not include nasal discharge among their signs and may or may not occur in the same horses at different times during their lives (Wood, 1999; Robinson, 2001). Nasal discharge was reported as a presenting clinical sign in only 50% of 50 horses with RAO referred to Edinburgh University in 1990 and 6% had discharge that was unilateral (McGorum, 1994).

In addition to EIPH, IAD, RAO and SPAOPD affecting the distal airways, various types of infectious pneumonia have nasal discharges among their presenting clinical signs (Hilbert *et al.*, 1980; Smith & Robinson, 1981; Pearson *et al.*, 1983; Falcon *et al.*, 1985; Knottenbelt, 1993; Raidal, 1995; Carr *et al.*, 1997; Wiegand & Schusser, 1997).

### *1.2.2.3 Risk factors for nasal discharge in respiratory disease of horses*

Age

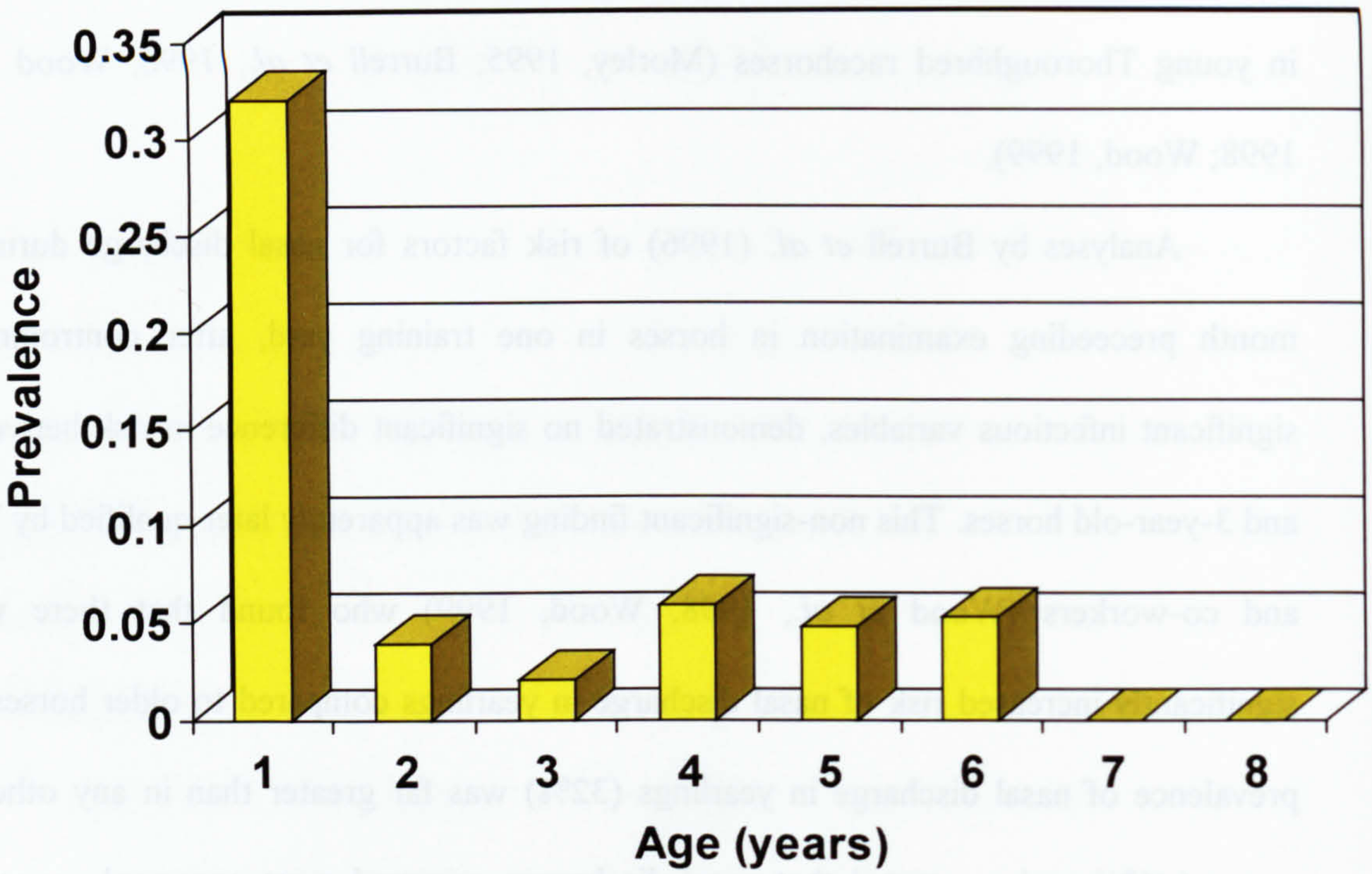
Age as a risk factor for the specific clinical sign of nasal discharge has not been widely examined with the exceptions of several longitudinal studies of respiratory disease in young Thoroughbred racehorses (Morley, 1995; Burrell *et al.*, 1996; Wood *et al.*, 1998; Wood, 1999).

Analyses by Burrell *et al.* (1996) of risk factors for nasal discharge during the month preceeding examination in horses in one training yard, after controlling for significant infectious variables, demonstrated no significant difference in risk between 2- and 3-year-old horses. This non-significant finding was apparently later qualified by Wood and co-workers (Wood *et al.*, 1998; Wood, 1999) who found that there was a significantly increased risk of nasal discharge in yearlings compared to older horses. The prevalence of nasal discharge in yearlings (32%) was far greater than in any other age group (<6%) and suggested that nasal discharges occurred most commonly soon after horses first entered training (Wood, 1999) (Figure 1.6, with permission from Wood *et al.* [1998]).

In studies of infectious upper respiratory tract disease, defined by mucopurulent nasal discharge and/or acute persistent coughing (these signs were not differentiated for separate analyses) in Thoroughbred racehorses in Saskatchewan, Canada, Morley (1995) found that horses younger than 4-years-old were at significantly increased risk than older animals of disease caused by influenza virus, EHV-4 and *S. equi*.

Although RAO is recognised to increase in prevalence with increasing age (Cook, 1976; McPherson *et al.*, 1979a) and the risk of nasal discharge associated with it may also be expected to increase with increasing age, the author is not aware of any data that specifically confirms this.

**Figure 1.6: Age-specific prevalence of nasal discharge during a longitudinal study of respiratory disease in Thoroughbred racehorses (from Wood *et al.* [1998])**




Infections

Among bacterial infections, marked bilateral, mucopurulent nasal discharges are frequently observed with *S. equi* (Ebert, 1969; Piche, 1984; Timoney & Powell, 1988; Dalgleish *et al.*, 1993; Hamlen *et al.*, 1994; Fintl *et al.*, 2000; Newton *et al.*, 2000b). Other bacterial species that have been associated with nasal discharges, particularly in young horses, include *S. zooepidemicus* (Mair & Lane, 1989; Hoffman *et al.*, 1993a; 1993b; Burrell *et al.*, 1994; Wood, 1999), *S. pneumoniae* (Huber *et al.*, 1988; Blunden *et al.*, 1991; 1994; Burrell *et al.*, 1994; 1996), *Pasteurella/Actinobacillus* spp. (Burrell *et al.*, 1994; Carr *et al.*, 1997), *Rhodococcus equi* (Smith & Robinson, 1981; Zinck *et al.*, 1986; Prescott *et al.*, 1991; Hoffman *et al.*, 1993c; Knottenbelt, 1993; Burks, 1996; Kolk *et al.*, 1999), *Bordetella bronchiseptica* (Cockram *et al.*, 1981; Bayly *et al.*, 1982; Vandevenne *et al.*, 1995) and anaerobic species (Sweeney *et al.*, 1984; 1985b; Mair &

Yeo, 1987; Sweeney *et al.*, 1991; Raidal, 1995; Burrell *et al.*, 1996; Racklyeft & Love, 2000).

Among viral infections, equine herpes viruses, including EHV-2, have been frequently associated with nasal discharge (Powell, 1975; Palfi *et al.*, 1978; Riveros *et al.*, 1978; Palfi *et al.*, 1979; Belak *et al.*, 1980; Fu *et al.*, 1986; Seahorn *et al.*, 1990; Adeyefa, 1992; Gilkerson *et al.*, 1994; McCartan *et al.*, 1995; Burrell *et al.*, 1996). Infection with influenza virus in naïve horses is frequently accompanied by profuse, mucopurulent nasal discharge (Gerber, 1970; Lucam *et al.*, 1974; Powell *et al.*, 1974b; Hannant *et al.*, 1988; Mumford *et al.*, 1990; Wood, 1991; Mumford *et al.*, 1994c; 1994d; Powell *et al.*, 1994; Morley, 1995; Newton & Mumford, 1995; Powell *et al.*, 1995; Gross *et al.*, 1998; Kastner *et al.*, 1999; Newton *et al.*, 1999a; Morley *et al.*, 2000; Newton *et al.*, 2000a). In populations of partially immune vaccinated horses where clinical signs are not necessarily typical of influenza, one of the most common signs is a rapidly spreading nasal discharge (Newton *et al.*, 1999a; Wood, 1999; Newton *et al.*, 2000a). In 2 longitudinal studies of respiratory disease in racehorses, infection with influenza virus has been strongly associated with nasal discharge (and other signs of upper respiratory tract disease) (Morley, 1995; Wood, 1999). Among other viral infections, nasal discharges have been observed with equine arteritis virus (Cole *et al.*, 1986; Timoney & McCollum, 1990; Johnson *et al.*, 1991; Holyoak *et al.*, 1993; McCollum *et al.*, 1995; Wood *et al.*, 1995; Paweska *et al.*, 1996; Paweska, 1997; Castillo Olivares *et al.*, 2001), equine rhinovirus (Plummer, 1962; Plummer & Kerry, 1962; Hofer *et al.*, 1973; Moraillon *et al.*, 1973; Powell, 1975; Studdert & Gleeson, 1978), equine adenovirus (Pascoe *et al.*, 1974; Kamada *et al.*, 1977) and parainfluenza-3 virus (Ditchfield *et al.*, 1963).

Organisms less frequently isolated from horses with signs of nasal discharge include *Pseudomonas* spp. (Govan *et al.*, 1992; Lal *et al.*, 1992), *Chlamydia psitacci* (Moorthy & Spradbrow, 1978; Burrell *et al.*, 1986a; Wills *et al.*, 1990) and *Cryptococcus*

*neoformans* (Hilbert *et al.*, 1980; Pearson *et al.*, 1983). Logistic regression modelling of nasal discharge in racehorses identified a significant association with non-felis glucose fermenting mycoplasmas in tracheal washes and a marginally significant association with ERV-1 infection (Wood, 1999).

Although respiratory infection of horses by the lungworm *Dictyocaulus arnfieldi* is rare and usually characterised by chronic coughing (Clayton & Murphy, 1980; Clayton & Duncan, 1981; Nielsen & Andersen, 1981; Fischer *et al.*, 1982; Lyons *et al.*, 1982; Goetz, 1984; Lester, 1993), nasal discharge may be observed in some cases (Round 1972; 1976; Lester, 1993).

Other factors

As discussed for pyrexia, transport of horses is frequently associated with signs of respiratory disease or so called 'shipping fever'. Maintaining horses with their heads and necks elevated for prolonged periods, such as when being transported, predisposes to accumulation of mucus and associated bacterial infection in the trachea, which may appear as a nasal discharge when horses subsequently lower their heads. It has been shown that experimental exposure of horses to levels of atmospheric ammonia equivalent to those found during transport caused marked nasal discharge and coughing (Katayama *et al.*, 1995).

Nasal discharge has been occasionally reported as an adverse reaction in several different veterinary interventions. Nasal discharge was reported shortly after vaccination in a small proportion of horses receiving influenza and/or tetanus vaccinations (Mair, 1988), although no definitive causal link between immunisation and development of clinical signs has yet been established (Dixon *et al.*, 1996). The potential of mucokinetic drugs in predisposing to nasal discharge was demonstrated in a report of an adverse reaction to clenbuterol in a 22-year-old horse receiving treatment for signs of RAO

(Gustin *et al.*, 1987). The horse developed nasal discharge, among other signs, shortly after administration of the $\beta_2$-adrenergic agonist. This may be explained because as well as causing bronchodilation through relaxation of smooth muscle, clenbuterol also increases mucociliary clearance by effectively thinning the mucus layer, improving the effectiveness of coughing and promoting secretion of water into the periciliary layer of the epithelial lining fluid (Dixon, 1992). Nasal discharge was observed with coughing in a report of pulmonary complications associated with maintaining horses for variable periods in flotation tanks (hydrotherapy) for treatment of skeletal injuries (McClintock *et al.*, 1986).

### 1.2.3 Coughing

Coughing is an inherent protective, reflex mechanism of the respiratory tract, which by its very occurrence may be taken to indicate some form of insult or abnormality. This section initially describes the cough reflex and then goes on to consider factors that are important in the occurrence of coughing in respiratory disease in horses.

#### *1.2.3.1 The cough reflex*

Coughing is a protective physiological mechanism for clearing foreign material from the larger conducting airways distal to the pharynx, particularly the larynx, trachea and bronchi (Dixon, 1992; Robinson, 1987; 1997c).

The cough reflex is initiated when irritant receptors in the airway epithelium of the larynx, trachea or bronchi are stimulated. These irritant receptors are most common in the airways just proximal and distal to the tracheal bifurcation at the carina (Robinson, 1987; 1997c) hence the need for administration of local anaesthetic during routine bronchoalveolar lavage (BAL) of the distal airways to reduce coughing prior to the passage of BAL tubing beyond the carina (Wehrli *et al.*, 2000). However, in a horse with

a sensitised larynx and/or trachea, for instance due to inflammation and/or infection, a cough reflex may be initiated by palpation of these structures externally (Mair, 1994).

Following stimulation of the irritant receptors, the neural signal passes along myelinated afferent fibres in the vagus nerve to the 'cough centre' in the medulla oblongata of the central nervous system (Karlsson *et al.*, 1988; Kenney & Divers, 1993). The efferent limb of the cough reflex passes along various different spinal nerves to complete inhalation and close the glottis, effectively sealing off the lower respiratory tract from the exterior. At the same time, pressure within the thorax is markedly increased by contraction of the expiratory muscles, particularly the abdominal muscles, resulting in compression of the air in the lungs and reduction in the cross-sectional area of the trachea and bronchi due to dynamic intrathoracic airway collapse. Coughing occurs when the glottis is opened and the compressed air in the lungs is expelled at high velocity through the constricted airways, blasting out foreign material to the pharynx and beyond (Robinson, 1987; 1997c). It is noteworthy that dynamic collapse of the airways, which only occurs within the thorax due to increased intrathoracic pressure from contraction of the expiratory muscles, means that coughing is less effective for clearing material from the extra-thoracic trachea which is not subject to dynamic collapse (Robinson, 1987). Therefore, the majority of routine lower airway endoscopy performed in studies of IAD in young horses (Burrell *et al.*, 1996; Christley *et al.*, 1999b; Wood, 1999; Chapman *et al.*, 2000) and in equine practice is generally restricted to the part of the airway that is least well cleared by coughing. This is probably fortunate in maximising the association of coughing and visible IAD.

Irritant receptors are stimulated when they are deformed by the presence of material on the epithelial surface, such as with accumulated secretions or foreign bodies, or by contraction of smooth muscle within the walls of the airways (i.e. with bronchoconstriction) (Robinson, 1987; 1997c). Therefore, cough is a clinical sign that is

associated with respiratory diseases producing impaired mucociliary clearance and consequent mucus accumulation, such as with IAD and RAO and/or with conditions resulting in bronchospasm, as with RAO. In these cases, coughing can be relieved by use of bronchodilator drugs. The sensitivity of the irritant receptors is increased by airway inflammation and pathological exposure of the epithelial surface, which is most commonly seen with viral infections such as equine influenza and herpes viruses (O'Niell *et al.*, 1984; Willoughby *et al.*, 1992; Sutton *et al.*, 1997). In conditions where mucociliary clearance is compromised, coughing becomes the most important mechanism for clearance of material from the airways and for this reason antitussive drugs that suppress coughing are not recommended for use in equine respiratory disease (Dixon, 1992). The consequences of losing the cough reflex were demonstrated in a 10-year-old mare with severe necrotising bronchopneumonia secondary to a melanoma in the caudal brainstem (Kenney & Divers, 1993). Loss of the cough reflex had been noted during endoscopy of the airways distal to the carina. Temporary loss of the cough and swallow reflexes was also noted as a post operative complication of left-sided prosthetic laryngoplasty in a Throughbred gelding due to probable surgical trauma to the cranial laryngeal nerve, which serves afferent sensory fibres in the larynx (Ahern, 1996).

### *1.2.3.2 Viral infections*

Equine influenza virus

The disease now known to be caused by equine influenza virus has long been recognised in completely naïve horses by characteristic coughing that is harsh and dry in nature, acute in onset and transmitted rapidly both within and between groups of susceptible animals (Gerber, 1970; Mumford *et al.*, 1990; Wood, 1991).

Equine influenza virus, as well as causing pathology of the upper respiratory tract, also extensively damages the ciliated epithelial cells lining the conducting airways. This

leads to disruption of normal mucociliary clearance with consequent accumulation of mucus and bacteria in the airways and exposure of the lamina propria and irritant receptors, all leading to frequent coughing (Lucam *et al.*, 1974; O'Niell *et al.*, 1984; Willoughby *et al.*, 1992; Sutton *et al.*, 1997). Figures 1.7a-c show a series of 3 scanning eletronmicrographs of equine tracheal ciliated epithelium at different stages of experimental influenza infection (courtesy of A. S. Blunden and J. A. Mumford).

Figure 1.7a shows normal ciliated epithelium in a non-infected animal. Figure 1.7b, from a horse 2 days after infection, demonstrates disruption of the cilia and adherence of bacteria. Figure 1.7c shows complete loss ciliated epithelium with exposure of the lamina propria in a horse 6 days after infection.

**Figure 1.7a: Scanning electronmicrograph of normal equine tracheal ciliated epithelium**

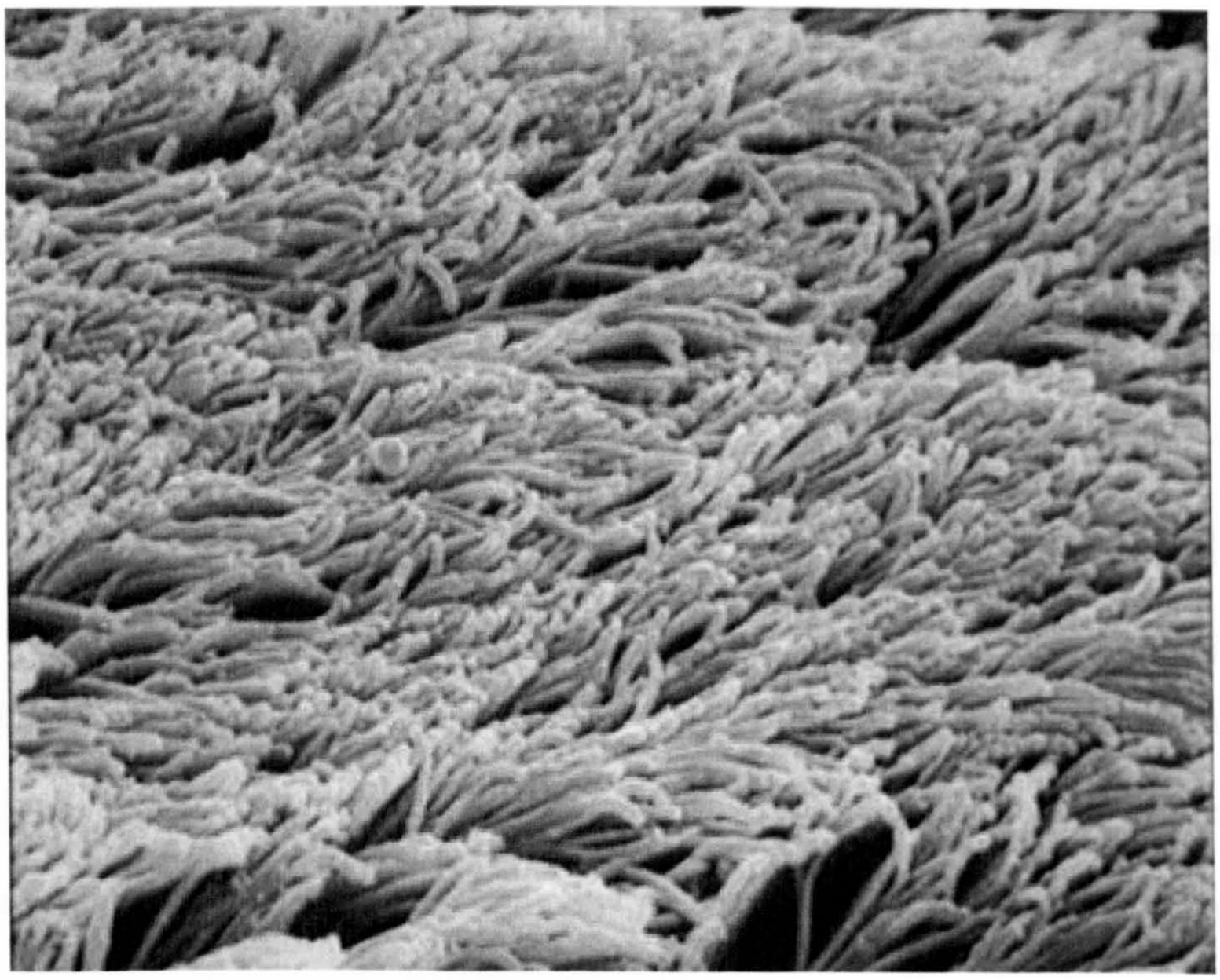


**Figure 1.7b: Scanning electronmicrograph of equine tracheal ciliated epithelium 2 days post-influenza infection**

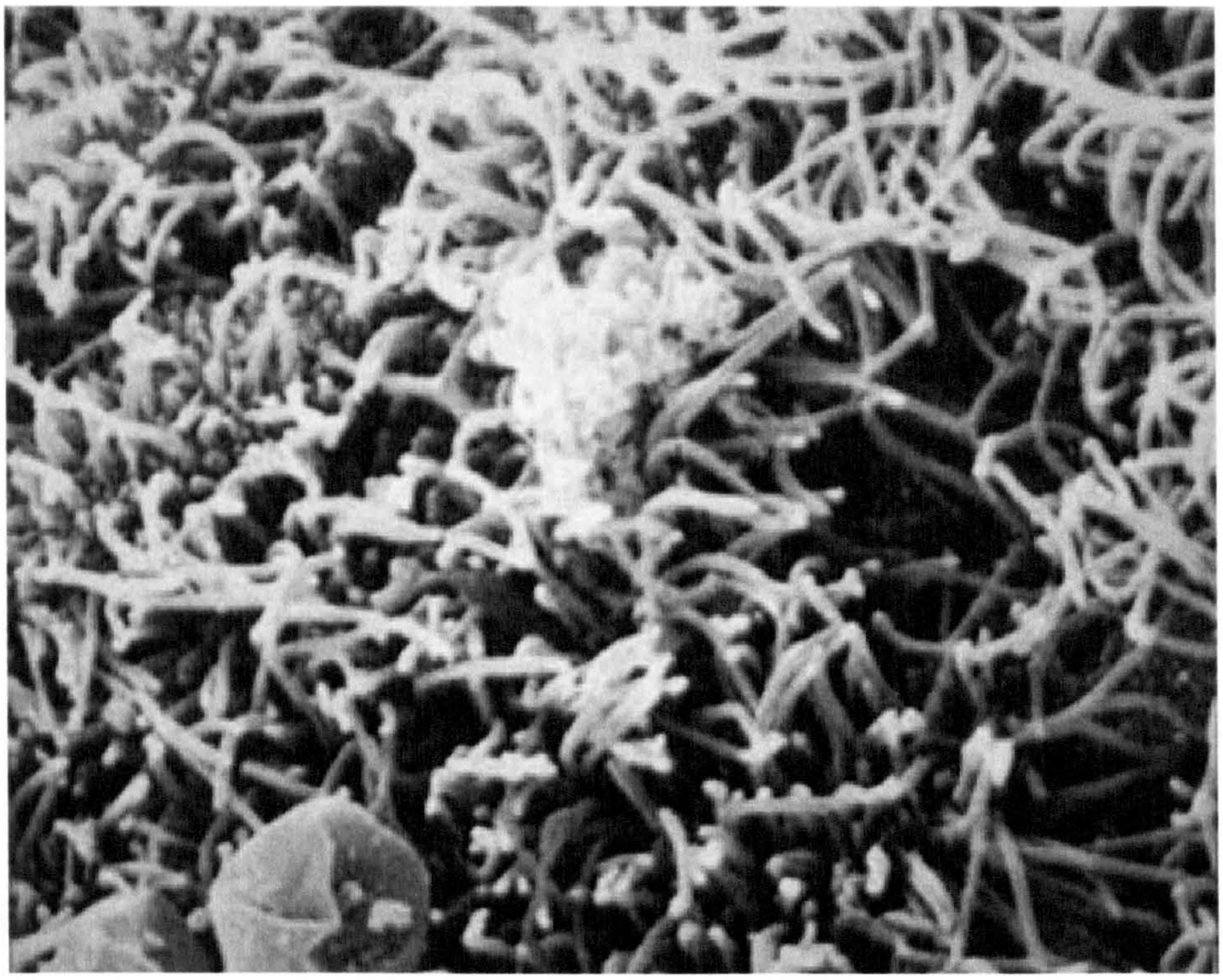


**Figure 1.7c: Scanning electronmicrograph of equine tracheal ciliated epithelium 6 days post-influenza infection**

Other viruses

Coughing may be observed as a clinical sign in a number of other viral respiratory infections of horses, including EHV-1 and EHV-4 (Powell, 1975; Allen & Bryans, 1986; Chong & Duffus, 1992; Mair, 1994; Morley, 1995; Mair, 1996c; Allen *et al.*, 1998; Sutton *et al.*, 1998), and less frequently with EHV-2 (Borchers *et al.*, 1998), ERV-1 (Plummer, 1962; Plummer & Kerry, 1962; Hofer *et al.*, 1973; Moraillon *et al.*, 1973) and EAV (Holyoak *et al.*, 1993). However, subclinical infections appear much more common with these infections than with influenza virus (Moraillon & Moraillon, 1978; Powell *et al.*, 1978; Studdert & Gleeson, 1978; Thomson, 1978; Gibson *et al.*, 1992; Gilkerson *et al.*, 1994; Wood *et al.*, 1995; Burrell *et al.*, 1996; Newton *et al.*, 1999b). A case control study was recently conducted in racehorses around Sydney, Australia, with cases defined as horses that coughed at least 4 times in a 10 minute period during exercise (Christley *et al.*, 2001b). This study failed to demonstrate any association between coughing and infection with adenovirus, ERV-1, ERV-2 or undifferentiated EHV-1/4, each diagnosed serologically.

### *1.2.3.3 Bacterial infections*

*Streptococcus equi*

Although not the most widely reported sign in equine strangles, infection with *S. equi* may be associated with a soft, moist cough that in most cases is likely to come from inflammation and irritation of the larynx and trachea from purulent discharges originating in the upper respiratory tract (e.g. Mair, 1994; Newton *et al.*, 2000b).

*Streptococcus pneumoniae*

Prior to investigations of respiratory disease in racehorses involving tracheal endoscopic examination there had been only a few reports of *S. pneumoniae* being associated with coughing in horses (Hofer *et al.*, 1973; Benson & Sweeney, 1984; Huber *et al.*, 1988).

Large numbers of *S. pneumoniae* ($10^6$-$10^8$ cfu/ml), either as pure culture or mixed with *S. zooepidemicus*, have been isolated from tracheal washes taken from coughing racehorses (Burrell *et al.*, 1986b; Mackintosh *et al.*, 1988; Burrell *et al.*, 1994). Consistent with this, intratracheal inoculation of 10ml of $10^9$ cfu *S. pneumoniae*/ml produced coughing among other clinical signs in experimental infections in Welsh mountain pony foals (Blunden *et al.*, 1991; 1994). More recently, the Australian case control study confirmed a significant dose-dependent association between *S. pneumoniae* in tracheal washes and coughing in racehorses (Christley *et al.*, 2001b).

*Streptococcus zooepidemicus* and *Pasteurella/Actinobacillus* spp.

The AHT and others have consistently demonstrated that *S. zooepidemicus* and *Pasteurella/Actinobacillus* spp. in tracheal washes are significantly associated with IAD in young racehorses (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood *et al.*, 1998; Wood, 1999; Chapman *et al.*, 2000). It has also been shown that coughing is a specific (84% of coughing horses had endoscopic and cytological evidence of IAD) but insensitive (only 38% of horses with IAD were coughing) indicator of IAD, particularly when the condition had been present for at least 2 months. Corroborating these findings, Christley *et al.* (2001) recently demonstrated a significant association between isolation of $>10^3$ cfu/ml of *S. zooepidemicus* or *Pasteurella/Actinobacillus* spp. from tracheal washes and coughing in Australian racehorses.

*Mycoplasma felis*

An outbreak of IAD, including coughing, that was associated with *M. felis* infection, occurred in a group of young Thoroughbred racehorses in Newmarket in November and December 1993 (Wood *et al.*, 1997a). Isolation in pure culture in some

animals and seroconversion in a large proportion of affected horses, was strongly suggestive of a primary pathogenic role for *M. felis* in this outbreak.

### *1.2.3.4 Pneumonia/pleuropneumonia*

Various types of infectious pneumonia and pleuropneumonia may present with clinical signs of coughing among other respiratory signs (Mair, 1989; Mair & Lane, 1989; Mair, 1991; Chaffin *et al.*, 1994b; Mair, 1994). Pleuropneumonia is an extension of pneumonia or pulmonary abscessation to involve the visceral pleura and pleural space as well as the pulmonary parenchyma (Chaffin & Carter, 1993). For the purposes of this discussion 'pneumonia' will be used from here on in to refer to both pneumonia and pleuropneumonia.

In foals infectious causes of pneumonia include *Rhodococcus equi* (Smith & Robinson, 1981; Falcon *et al.*, 1985; Varga *et al.*, 1997) and various other bacterial species including *Streptococcus* spp. (Benson & Sweeney, 1984; Mair, 1989; Chaffin & Carter, 1993). Inhalation pneumonia may occur in foals secondary to congenital upper respiratory tract abnormalities (Stick & Boles, 1980; Riley *et al.*, 1991).

Coughing with pneumonia, associated with infection with various different bacterial species, occurs secondary to influenza virus infection (Lucam *et al.*, 1974; Smith, 1979), as a complication of *Streptococcus equi* infection known as 'bastard strangles' (Rooney, 1979; Ford & Lokai, 1980; Sweeney *et al.*, 1987b) and following long-distance transport (Oikawa *et al.*, 1994; 1995). Inhalation of food, saliva and other material frequently results in acute coughing and may culminate in pneumonia. This may occur, for example, because of oesophageal obstruction ('choke') or dysphagia of various causes (Mair, 1994).

In racehorses it has been suggested that blood in the airways following intensive exercise (EIPH) may provide an ideal medium for bacterial replication and therefore

predispose to pneumonia (Raphel & Beech, 1982). Apparent differences in susceptibility to pneumonia between Thoroughbreds (increased risk) and Standardbreds (reduced risk) in America have been partially attributed to differences in prevalence of EIPH between these 2 breeds (Austin *et al.*, 1995). However, EIPH as an underlying factor in pneumonia is somewhat refuted by marked geographical differences in prevalence of pneumonia in racehorses between the UK and North America (Mair & Lane, 1989; Chaffin & Carter, 1993) as EIPH is highly prevalent in both areas of the world (Clarke, 1985; Chaffin & Carter, 1993). It has been postulated that the reason for increased pneumonia among North American racehorses is more likely to be due to inhalation of kicked back track material while racing on 'dirt', which is common practice in America but relatively uncommon in the UK (Chaffin & Carter, 1993). In addition, it has been shown that a single bout of high intensity exercise contributes to increased bacterial contamination of the lower respiratory tract in otherwise healthy Standardbred horses (i.e. no signs of lower airway disease) undergoing treadmill exercise (Raidal *et al.*, 1997b). However, these increased infectious burdens were rapidly cleared in these horses, which did not develop subsequent lower airway disease and the authors postulated that inhaled track debris and blood from EIPH may be additional contributory factors in overwhelming mucociliary clearance and predisposing to pneumonia in racehorses (Raidal *et al.*, 1997b).

*1.2.3.5 Lungworm infection*

The adult nematode *Dictyocaulus arnfieldi* resides in the bronchi and can be a cause of coughing in horses (Andersen & Fogh, 1981; Clayton & Duncan, 1981; George *et al.*, 1981; Nielsen & Andersen, 1981; Fischer *et al.*, 1982; Lyons *et al.*, 1982; Poncet, 1983; Goetz, 1984; Whitwell & Greet, 1984; Lester, 1993; McGorum, 1994; Dixon *et al.*, 1995b; Ramachandran, 1995; Burks, 1998), a disease similar to parasitic bronchitis or 'husk' in cattle (Urquhart *et al.*, 1987).

*D. arnfieldi* infection is ubiquitous in donkeys, which are believed to be the natural reservoir host for the parasite, causing very few clinical signs. Without use of anthelmintics donkeys acquire infection as foals and tend to remain infected all their lives because with patent infections in this species there is frequent re-exposure to $L_3$ stage larvae on pasture. Infection in horses is much less common as the infection is not patent and therefore requires co-grazing with donkeys, usually in the late-summer or autumn months. It is believed that *Pilobilus* fungi may play a role in disseminating infectious larvae away from faeces and onto the surrounding pasture as occurs with *D. viviparus* in cattle (Urquhart *et al.*, 1987). Treatment of lungworm infection with ivermectin or benzimadazoles is straightforward but may elicit violent coughing for several days after treatment as the killed parasites are expelled (Fischer *et al.*, 1982).

*1.2.3.6 Upper respiratory tract conditions*

A number of conditions of the upper respiratory tract may be associated with clinical signs of coughing, particularly involving the epiglottis and/or soft palate. These conditions include soft palate ulceration (Norwood, 1983; Gille & Lavoie, 1996), epiglottitis (Hawkins & Tulleners, 1994), soft palate hypoplasia (Riley *et al.*, 1991), epiglottic entrapment (Honnas & Wheat, 1988; Honnas *et al.*, 1990; Lumsden *et al.*, 1994) with or without dorsal displacement of the soft palate (Haynes 1981; Tate *et al.*, 1990) and subepiglottic cysts (Stick & Boles, 1980).

Neoplasia (Boulton, 1985; Hilbert *et al.*, 1988; Tuckey *et al.*, 1995; Savage, 1998; Scarratt & Crisman, 1998; Tremaine & Dixon, 2001), including progressive ethmoid haematoma (Cook & Littlewort, 1974; Tremaine & Dixon, 2001) or foreign bodies (Arvidsson, 1981; Urquhart *et al.*, 1981; Brown & Collier, 1983; Duckett *et al.*, 1983; Mair, 1987; Scheidemann *et al.*, 1993) in the upper respiratory tract may present with coughing.

Not surprisingly, coughing may also be a common sign following different types of surgery of the upper airway, particularly of the larynx in horses with left sided laryngeal hemiplegia (Huskamp, 1980; Speirs, 1986; Tulleners *et al.*, 1988; Hay *et al.*, 1993; Holcombe *et al.*, 1994; Russell & Slone, 1994; Hawkins *et al.*, 1997).

### *1.2.3.7 Environmental factors*

Allergic lung disease (RAO/SPAOPD)

Chronic and persistent coughing is frequently observed with the allergic respiratory diseases of recurrent airway obstruction (RAO) and summer pasture associated obstructive pulmonary disease (SPAOPD) (Cook, 1976; Robinson, 1987; Seahorn & Beadle, 1993; McGorum, 1994; Seahorn & Beadle, 1994; Mair, 1996b; Robinson *et al.*, 1996; Robinson, 1997c; McGorum & Dixon, 1999; Robinson, 2001).

The common underlying pathological process behind both RAO and SPAOPD is a pulmonary hypersensitivity to different inhaled allergens (Cook, 1976; Derksen *et al.*, 1997; Robinson *et al.*, 1996). The notable differences between RAO and SPAOPD in seasonality and more specifically, the apparently dichotomous risk factors of housing predisposing to RAO and grazing to SPAOPD, are probably attributable to differences in the source of allergens to which horses are sensitive.

RAO is triggered by exposure to inhaled organic dusts, usually from poor quality straw and hay which contain a mixture of species of moulds, spores, forage mites and endotoxins (McPherson *et al.*, 1979b; Derksen *et al.*, 1988; McGorum *et al.*, 1993c; Robinson *et al.*, 1996; McGorum *et al.*, 1998; Robinson, 2001). Results from several inhalation challenge studies conducted to identify important specific triggers (e.g. allergens/endotoxin) and controlled against natural challenge with exposure to hay/straw, suggest that naturally-occurring RAO is attributable to multiple factors within the environment (McGorum *et al.*, 1993b; 1993c; Robinson *et al.*, 1996; Pirie *et al.*, 2001a;

Robinson, 2001). The responses observed in extract challenges were uniformly less than those that occurred with exposure to hay/straw.

In SPAOPD the allergens are believed to be moulds on grasses (Seahorn & Beadle, 1993; Derksen *et al.*, 1997; Robinson *et al.*, 1996) or inhaled pollens (McGorum, 1994). The nature of allergens associated with SPAOPD has not been definitively determined, although some differences in nasal fungal isolates have been demonstrated between horses kept at pasture and those kept in stables (Seahorn & Beadle, 1994). There are anecdotal suggestions that exposure to the pollen of oil seed rape (various *Brassica* species), an increasingly ubiquitous summer field crop in the United Kingdom, is the underlying cause of respiratory disease in horses exposed to it (Dixon & McGorum, 1990; Mair, 1996b). However, preliminary investigations have suggested that although exposure to oil seed rape pollen may exacerbate underlying respiratory problems, it is not itself a significant primary cause of SPAOPD (McGorum & Dixon, 1992; McGorum, 1994).

Coughing with RAO and SPAOPD is most likely due to stimulation of irritant receptors in the epithelium of the conducting airways through several coinciding and probably synergistic mechanisms. These include chemical and physical irritation from inhaled foreign/allergen material found in the poor environments that predispose to these conditions (i.e. direct, non-immunogenic effects of stable dust) as well as bronchoconstriction, mucus hypersecretion and consequent mucus accumulation through impaired mucus clearance (Robinson, 1987; Robinson *et al.*, 1996 Robinson, 1997c; 2001).

Other environmental factors

As previously discussed for pyrexia with respiratory disease, horses undergoing long distance travel may be at increased risk of coughing (Oikawa *et al.*, 1994; 1995),

which may be due to a combination of mucus accumulation, primarily as a result of prolonged raised head carriage and exposure to increased levels of toxic gases such as ammonia (Katayama *et al.*, 1995).

Epidemiological studies are providing increasing evidence for a link between air pollution and respiratory disease in man, but although there are anecdotal reports that RAO is more prevalent in suburban than rural environments, to date there are no studies that confirm a similar association in horses (Mair, 1995).

Acute onset coughing may be precipitated in racehorses during or immediately following racing due to inhalation of foreign track material kicked back from other horses further ahead in the field (Arthur, 1983; Sweeney *et al.*, 1991; Chaffin & Carter, 1993; Austin *et al.*, 1995). This occurs particularly in horses using so-called 'all weather' or 'dirt' surfaces, which in the UK are of various types and comprise a mixture of sand and water retardant materials. As already discussed, inhalation of and failure to clear inhaled 'kickback' from the lungs has been implicated in causing pleuropneumonia in racehorses in North America (Arthur, 1983; Chaffin & Carter, 1993; Austin *et al.*, 1995; Raidal, 1995; Raidal *et al.*, 1997b). The effects of kickback may be worse in the USA than in the UK because of the looser nature of the track surface. 'All weather' surfaces in the UK tend to be petroleum based for water resistance and consequently have reduced 'kickback' compared to the drier surfaces used in warmer climates. There may also be additional effects by longer distance travel in North America (Mair & Lane, 1989; Austin *et al.*, 1995).

### *1.2.3.8 Other factors*

Age

Coughing in young racehorses, as with nasal discharge, is most prevalent in younger animals. In North America coughing in conjunction with the presence of nasal

discharge (defined as cases of infectious upper respiratory disease; IURD) was inversely associated with age where horses were infected with influenza virus, equine herpesvirus or *S. equi* (Morley, 1995). A negative association between age and coughing was also demonstrated in horses with bacterial lower respiratory tract disease in Australia, in the absence of equine influenza virus infection (Christley *et al.*, 1999b; 2001a).

Training and racing

The case control study conducted in Australia by Christley, using coughing as a clinical indicator of equine respiratory disease, demonstrated that this sign was most prevalent in horses in the early stages of training (Christley *et al.*, 1999b; 2001a). This was consistent with coughing being least prevalent in those animals that had been in the training yards for the longest time. Data from this study also suggested, having controlled for the effects of age, stage of training and time since last transported, that racing within the previous 7 days was associated with an increased risk of coughing compared to horses that had never previously raced. There was also evidence that horses that had been transported at least 14 days previously were at increased risk of coughing compared to those transported less than one week previously. As the authors stated, this appeared somewhat anomalous as transport is recognised as a risk factor for respiratory disease (Raphel & Beech, 1982; Austin *et al.*, 1995; Raidal *et al.*, 1997a). However, taking into account other significant variables in their analyses, this probably indicated a lag period between recent arrival in the training yard and the onset of coughing.

In the Canadian study of IURD, the effect of previous racing, controlling for other significant factors, demonstrated a reduction in odds of disease (Morley, 1995). This indicated that for influenza related IURD, previous racing apparently conveyed a protective effect over and above that conveyed by the effects of increased age, male gender and raised antibody titres which had been identified as significant risk factors in

this particular study. The author speculated that this was most likely evidence of a 'healthy worker' effect, i.e. only horses without clinical signs of disease would be sent racing rather than that the act of racing somehow conveyed protection.

Miscellaneous

Other miscellaneous causes of coughing in horses include blood in the airways with EIPH (McGorum, 1994) and physical tracheal abnormalities including collapse, usually in aged ponies, (Mair & Lane, 1990; Mair, 1994; McGorum, 1994) and more rarely obstruction from intraluminal masses (Wenger & Caron, 1988). Other rare causes are thoracic neoplasia (Mair & Brown, 1993; McGorum, 1994), Crofton weed (*Eupatorium adenopharum*) toxicity in Australia (O'Sullivan, 1979), eosinophilic interstitial pneumonia (Dixon *et al.*, 1992; Carrick, 1994), pulmonary infarction or oedema (Carr *et al.*, 1997), and cardiac diseases such as rupture of the mitral chordae tendinae or bacterial endocarditis (Mair, 1994; Reef *et al.*, 1998).

### 1.2.4 Dyspnoea

Dyspnoea is the medical term used to describe the clinical presentation of laboured or exaggerated breathing and is principally an indication of inadequate ventilation and/or insufficient oxygenation of the circulating blood (Blood & Studdert, 1988). Physiologically, dyspnoea attempts to correct these insufficiencies and so clinically it may be manifested as an elevated respiratory rate (tachypnoea), exaggerated effort by intercostal and/or abdominal muscles ('abdominal effort' or 'heaves'), flaring of the nostrils, extension of the head and neck, elbow abduction and abnormal sounds (usually stridor) (Mair & Lane, 1996).

Dyspnoea may be thought of as having 4 classic subdivisions (Mair & Lane, 1996). These are i) obstruction, ii) reduced thoracic capacity, iii) compromised gas

exchange and iv) physiological disorders. Table 1.1, adapted from Mair (1996), summarises conditions that cause dyspnoea in the horse according to each of these 4 subdivisions. Some of these conditions, such as RAO, SPAOPD and pneumonia, may be classified in more than one category as they cause reduced gas exchange but are also 'obstructive' diseases.

In the majority of cases of dyspnoea there is likely to be both an increased rate and depth of respiration, with the effort used for both the inspiration and expiration phases of breathing being exaggerated to some extent (Mair & Lane, 1996). However, depending on the cause of dyspnoea or site of obstruction, either inspiratory or expiratory efforts may be more pronounced than the other. For example, upper respiratory tract (and by definition extra-thoracic) obstructions will tend to produce more pronounced *inspiratory* compromise and hence require additional effort at this stage of the breathing cycle. This is because respiratory obstruction will be exacerbated by dynamic airway collapse due to the reduced intraluminal pressure that occurs at this stage of respiration. Examples of this have been observed in horses with upper respiratory tract obstruction of various types. These include intraluminal tracheal obstruction (Wenger & Caron, 1988), tracheal compression by peri-tracheal abscess (Tessier *et al.*, 1996), choanal atresia (Richardson *et al.*, 1994a), persistent DDSP in foals (Altmaier & Morris, 1993), arytenoid chondritis (Haynes *et al.*, 1980) and abnormalities of the nostrils (El Maghraby, 2000), false nostrils (Torre *et al.*, 1993) and larynx (Haynes *et al.*, 1980; Duncan & Brook, 1985; Muylle, 1988; Behrens & Pinero, 1990; Burba *et al.*, 1991; Dixon *et al.*, 1993b).

**Table 1.1: Subdivision of causes of dyspnoea in the horse (adapted from Mair [1996])**

| Obstruction | Reduced thoracic capacity | Compromised gas exchange | Physiological disorders |
|---|---|---|---|
| *Upper respiratory tract (URT)* | | | |
| Alar cartilage/false nostril abnormality | Diaphragmatic rupture or hernia | Paraquat poisoning | Carbon monoxide poisoning |
| Arytenoid chondropathy | Pleural effusion | Pneumonia/pleuropneumonia (various) | Circulatory shock/reduced tissue perfusion |
| Congenital abnormality (choanal atresia) | Pneumothorax | Pulmonary emphysema | CNS respiratory centre disorders |
| Epiglottitis | Pyothorax | Pulmonary oedema/smoke inhalation | Excessive environmental heat/humidity |
| Guttural pouch empyema or tympany | Respiratory pain (e.g. rib fracture) | Recurrent airway obstruction (RAO) | Metabolic acidosis |
| Intraluminal tracheal obstruction | Thoracic musculature dysfunction | SPAOPD** | |
| Laryneal paralysis or oedema | | | |
| Nasopharyngeal/tracheal foreign body | | | |
| Para-pharyngeal abscessation | | | |
| Peri- tracheal abscess/mass | | | |
| Persistent DDSP* in foals | | | |
| Pharyngeal paralysis | | | |
| Proximal oesophageal obstruction | | | |
| Retropharyngeal lymphadenopathies | | | |
| *S. equi* infection ('strangles') | | | |
| URT neoplasia (various) | | | |
| *Lower respiratory tract (LRT)* | | | |
| LRT neoplasia (various) | | | |
| Lungworm | | | |
| Pneumonia/pleuropneumonia (various) | | | |
| Pulmonary oedema/smoke inhalation | | | |
| Recurrent airway obstruction (RAO) | | | |
| SPAOPD** | | | |
| Viral/bacterial hypersensitivity | | | |

*Dorsal displacement of the soft palate ** Summer pasture associated obstructive pulmonary disease

In contrast, obstructions of the lower, intrathoracic respiratory tract (e.g. severe IAD, pneumonia, RAO, and SPAOPD) will produce more obvious *expiratory* dyspnoea because there is dynamic collapse of these airways during expiration when intraluminal airway pressure is exceeded by extraluminal, pleural pressure (Mair & Lane, 1996; Robinson *et al.*, 1996; Robinson, 1997c). This results in the animal adopting a breathing strategy that allows it to exhale most of its tidal volume at an early stage in expiration which is then followed by an abdominal push that forces a relatively small volume of air through the obstructed and compressed airways (Robinson *et al.*, 1996; Robinson, 1997c).

Obstructions of the larger diameter upper airways, because their total cross sectional area is actually much smaller than that of the distal airways, may cause dyspnoea relatively easily (Robinson, 1997a). In order for obstructive lower respiratory tract diseases to cause signs of dyspnoea, obstruction of the bronchioles must be both diffuse and severe, i.e. a large proportion of the total cross sectional area of these airways must be obstructed (Robinson, 1997a).

Inspiratory dyspnoea may also occur in diseases of the lower respiratory tract that inhibit expansion of the lungs, therefore requiring additional inspiratory effort to try and force air into the gas exchange regions (Mair & Lane, 1996). This type of dyspnoea occurs with interstitial pneumonia and alveolitis (Prescott *et al.*, 1991; Whitwell, 1992; Ainsworth *et al.*, 1993; Kelly *et al.*, 1995) and restrictive diseases affecting the pleura, including pneumothorax (Boy & Sweeney, 2000), pyothorax/pleural effusion (Smith, 1977; Raphel & Beech, 1982; Ogilvie *et al.*, 1983; Collins *et al.*, 1994; Carr *et al.*, 1997; Scarratt *et al.*, 1997; Quintavalla, 1998) and ruptured diaphragm (Perdrizet *et al.*, 1989; Ewart *et al.*, 1992; Goehring *et al.*, 1999).

### 1.2.5 Lymph node enlargement

The respiratory tract in the horse has associated lymphoid tissue throughout, which comprises series of multiple aggregated lymph nodes organised as anatomically discrete lymphocentres (Getty, 1975). The main equine lymphocentres relating to the respiratory tract are, from the nostrils caudally; the submandibular (SMLN or mandibuar), parotid, retropharyngeal (RPLN), superficial and deep cervical, mediastinal, axillary, dorsal and ventral thoracic, and bronchial (Getty, 1975). The vast majority of these lymphocentres, with the possible exception of the more superficial submandibular chain of nodes, are not normally palpable.

Lymph node enlargement in association with respiratory disease in horses is most frequently seen with *S. equi* infection, with the disease colloquially referred to as 'strangles' because of the respiratory obstruction caused by lymph node abscessation in more severe cases. *S. equi* infection may affect all the lymphocentres of the head (submandibular, parotid and retropharyngeal), causing swelling, pain, abscessation and consequent disturbance to eating, swallowing and breathing. If left untreated lymph node abscesses usually discharge their purulent contents as a normal part of the course of the disease. In the case of submandibular lymph nodes there will be external discharge through the skin between the rami of the mandibles and for the parotid lymphocentre a discharge will be seen on the side of the face just below the ear (Sweeney, 1996). However, the RPLNs more commonly discharge internally through the ventral floor of the guttural pouch resulting in guttural pouch empyema, which when this drains via the pharyngeal pouch opening may be swallowed or appear as a purulent nasal discharge (Knight *et al.*, 1975; Sweeney, 1996; Newton *et al.*, 1997b; Fintl *et al.*, 2000). As mentioned for the derivation of the name 'strangles', another major clinical sign associated with lymph node abscessation is dyspnoea due to physical respiratory obstruction, which may occur through occlusion of the pharynx

and larynx by the RPLNs or cranial deep cervical nodes or the trachea by the caudal cervical (Little *et al.*, 1985), mediastinal (Rigg *et al.*, 1985) or bronchial lymph nodes.

Such a case of obstruction by peritracheal abscessation has also been reported associated with *S. zooepidemicus* infection of the caudal cervical lymph nodes (Tessier *et al.*, 1996). Lymph node enlargement with or without abscessation may be observed with other bacterial infections including *Rhodococcus equi* (Roberts *et al.*, 1980; Vyslouzil *et al.*, 1984; Zinck *et al.*, 1986), *S. pneumoniae* (Blunden *et al.*, 1994) and *Actinobacillus* spp. (Zaharija *et al.*, 1979). Viral infections, including EHV-1 (Allen & Bryans, 1986; Kydd *et al.*, 1994; Heldens *et al.*, 2001) and ERV-1 (Plummer, 1962; Plummer & Kerry, 1962; Hofer *et al.*, 1973), may also result in lymph node enlargement.

### 1.2.6 Scoring of clinical disease

Clinical scoring has been used in a number of studies of equine respiratory disease and these vary as to the range of signs that are assessed and the relative weights assigned to these individual parameters.

There have been several studies that used clinical scoring on aspects of allergic respiratory disease such as RAO and SPAOPD. In some of these studies particular attention was paid to assessing the nature of the respiratory effort and this was done in a number of ways. In some studies this was by only scoring the extent of nasal flaring and abdominal lift (Seahorn *et al.*, 1997; Rush *et al.*, 1998; Costa *et al.*, 2000; Robinson *et al.*, 2000), which were given equivalent weighting. In other studies of RAO a wider range of clinical parameters were assessed. Tesarowski *et al.* (1996) used abdominal lift and nasal flaring in a complex 25-point scale, based on a system described by Hoffman *et al.* (1992b), that also included coughing, nasal discharge, respiratory rate, thoracic auscultation and percussion, and various sounds such as wheezes and crackles from the trachea, bronchi and lungs. In an assessment of the role of endotoxin in RAO, Pirie *et al.* (2001b) used a 13-point scale that

included coughing, nasal discharge, dyspnoea, respiratory rate, thoracic auscultation, pulse rate and pyrexia, with respiratory rate and dyspnoea being weighted the most heavily. In studies of airway responsiveness and its attenuation Hare and co-workers described a 15-point scale based on cough, nasal discharge, respiratory rate, thoracic auscultation, bronchoscopy, expiratory effort and SMLN enlargement (Hare *et al.*, 1994; Hare & Viel, 1998; Hare *et al.*, 1999).

A number of studies of equine viral diseases including trials of vaccine efficacy have used clinical scoring systems, usually applied to much younger horses than in RAO studies and with less emphasis on characterising the degree of respiratory effort. The scoring systems adopted in these studies have been generally more consistent in the range of signs that are evaluated, although the weighting of these has differed.

In a study of the efficacy of an inactivated EHV vaccine, Heldens *et al.* (2001) used a 29-point scale which included demeanour, appetite, respiratory rate, nasal and ocular discharge, coughing and SMLN enlargement. In this system, death or paralysis contributed 10 points in the demeanour score and severe mucopurulent nasal and ocular discharges each scored 4.5 points. In a study of the effect of exercise on severity of signs in influenza infected horses, (Gross *et al.*, 1998) used a 6-point score that comprised equally weighted single scores each for fever, inappetance, depression, coughing, mucopurulent nasal discharges and abnormal lung sounds. In studies of a modified live equine influenza virus vaccine, Chambers *et al.* (2001) and Townsend *et al.* (2001) used a 7-point scale that scored coughing, nasal discharge, abnormal respiration and depression characterised by lethargy and inappetance. This score was most heavily weighted towards nasal discharge, which scored a possible maximum of 3 points and was least weighted towards abnormal respiration and depression which each scored a single point if observed.

Many other studies, particularly on equine influenza virus infections, have evaluated a similar range of signs by allocation of scores for severity to each sign but rather than

generating aggregated summary scores for overall respiratory disease, they have evaluated each sign individually (Mumford *et al.*, 1983; 1988; 1990; Hoffman *et al.*, 1993a; Burrell *et al.*, 1994; Mumford *et al.*, 1994b; 1994c; 1994d; Burrell *et al.*, 1996; Kastner *et al.*, 1999; Morley *et al.*, 1999; Newton *et al.*, 1999b; Morley *et al.*, 2000)

In the majority of descriptions of scoring systems used to evaluate clinical severity of respiratory disease in horses the relative weighting assigned to different signs generally remains unjustified. However, it is usually evident from the scores used which signs are considered the most important by the researchers involved although it is frequently not clearly stated that the scoring system was adopted prior to the conduct of the study and was not applied to data *post hoc.*

## 1.3 Subclinical respiratory disease in horses

Respiratory disease in horses may not necessarily be accompanied by overt clinical signs of disease as there may be pathology of the airways that remains effectively subclinical unless additional diagnostic investigations are carried out.

### 1.3.1 Haematological investigations

Although some veterinary surgeons and racehorse trainers hold store by the diagnostic benefit of haematological investigations (Allen & Frank, 1982; Mason *et al.*, 1989a; 1989b; 1990), if changes to haematological parameters are detected these are often non-specific and may be seen in healthy as well as diseased individuals (Wood, 1999). The use of haematology in the diagnosis of subclinical respiratory disease is not discussed further here.

### 1.3.2 Respiratory endoscopy

The advent of more widespread use of fibreoptic endoscopy in the examination of the respiratory tract of horses allowed hitherto inaccessible areas of the equine respiratory system to be assessed with respect to pathological conditions (Cook, 1974c; Burrell, 1985). As well as demonstrating that the lung was the source of bleeding for epistaxis (Cook, 1974a) and that such bleeding (EIPH) was actually far more prevalent than epistaxis (Pascoe & Wheat, 1980, Pascoe & Raphel, 1982) because blood was more frequently swallowed than appeared at the nostrils, fibreoptic endoscopy revealed other 'pathological' phenomena of the equine respiratory tract.

#### *1.3.2.1 Pharyngeal lymphoid hyperplasia (PLH)*

One such 'pathological' condition was the presence of hyperplastic lymphoid follicles in the walls of the pharynx (pharyngeal lymphoid hyperplasia; PLH), particularly in young horses. PLH has been i) attributed to specific viral infections (Prickett, 1969; Blakeslee *et al.*, 1975), ii) cited as a reason for poor performance in some horses (Raker & Boles, 1978) and iii) observed to reduce following vaccination (Montgomery, 1981). However, a subsequent study in racehorses failed to demonstrate any significant associations between PLH and i) isolation of EHV-2 from the nasopharynx, ii) racing performance and iii) antibody titres raised to EHV-1 by vaccination (Burrell, 1985). This study did, however, clearly demonstrate a significant inverse correlation between decreasing severity of PLH and increasing age and time spent in the training yard, which was consistent with earlier age-related findings (Raphel, 1982). It is now generally accepted that this condition is a normal physiological phenomenon in young horses (Embertson, 1997).

### *1.3.2.2 Endoscopically visible tracheal mucus*

In addition to examination of the upper respiratory tract, fibreoptic endoscopy has facilitated the examination of the airways distal to the larynx, particularly the distal trachea, allowing visible assessment of the presence of mucus (and/or blood) at this site (Burrell, 1985). Different groups have assessed the degree of mucus in the trachea using different scoring systems. One of the most widely adopted systems has been the 3-point score described by Burrell (1985) where grade 0 was no mucus observed, grade 1 was isolated globules, grade 2 was a thin continuous stream <15mm wide and grade 3 was a thick continuous stream >15mm wide (Burrell, 1985; Wood *et al.*, 1993a; Burrell *et al.*, 1994; 1996; Wood *et al.*, 1997a; Wood, 1999; Chapman *et al.*, 2000). Others have used/use a similar system but based on a 5-point scale (Dixon *et al.*, 1995b; Holcombe *et al.*, 2001) (M. Shepherd and B. Herinckx – personal communications). The 5-point scoring system was described by Holcombe *et al.*, (2001) as follows: 0 – no visible mucus, 1 – singular small blobs, 2 – multiple blobs only partly confluent, 3 – ventrally confluent, 4 – large ventral pool and 5 – profuse amounts occupying >24% of the tracheal lumen. Recently, a more complex scoring of tracheal endoscopy findings has been described with scores ranging between 2 and 20 (Hamm *et al.*, 2002). This system took 6 parameters into account : i) amount ii) nature and iii) location of discharge, iv) whether or not there was discharge visible on insertion and v) withdrawal of the endoscope, and vi) clinician's overall rating.

The timing of examination relative to exercise would appear to be an important factor in whether or not mucus is visible in the trachea at endoscopy (Burrell, 1985; Lumsden *et al.*, 1995; Wood, 1999). Several studies have concluded that endoscopy conducted after exercise increases the likelihood of observing tracheal mucus. Burrell (1985) observed that the prevalence of tracheal mucus was significantly greater ($P<0.001$) following cantering or galloping (62%) than after walking, trotting or no exercise (16%). In a series of referred cases of suspected upper respiratory tract disorders, 20% of horses were

only found to have increased levels of tracheal mucus following exercise (Lumsden *et al.*, 1995). It is therefore important in critically considering the findings of studies using tracheal endoscopy of horses, to take into account whether or not the procedure has been conducted after exercise or not. In this thesis, in the study of respiratory disease in Thoroughbred racehorses, endoscopy was conducted after exercise but in the Welsh Mountain ponies endoscopy was performed following only the exercise required to bring the animals from the field to the adjacent examination area.

*1.3.2.3 Collection of tracheal aspirates*

As well as visible assessment of mucus and/or blood throughout the length of the trachea, endoscopy also facilitates collection of respiratory samples by instillation and aspiration of sterile fluid via catheter placed in the biopsy channel of the endoscope (Greet, 1982; Whitwell & Greet, 1984; Dixon, 1995). Alternatively, collection of tracheal aspirates from horses may be conducted by transtracheal catheterisation techniques (Mansmann & Knight, 1972; Beech, 1981; Greet, 1982; Dixon, 1995). Although it avoids sample contamination by bypassing the upper respiratory tract, transtracheal aspiration does have some disadvantages compared to the transendoscopic technique. Transtracheal aspiration can be considered surgically invasive and as such requires good aseptic conditions to avoid subcutaneous infection (Dixon, 1995; Greet, 1982). Samples may become contaminated by blood from the stab incision and care is needed not to cut the catheter during withdrawal through the needle that is used to access the tracheal lumen through the skin (Dixon, 1995; Greet, 1982; Sweeney *et al.*, 1989). The procedure is also relatively time consuming, may require sedation of the horse to avoid undue movement and has limitations with respect to being frequently repeated. In addition, it has been shown that the degree of bacterial contamination from negotiating the upper respiratory tract in transendoscopically collected samples is not usually large (Sweeney *et al.*, 1989; Darien *et al.*, 1990; Christley *et al.*,

1999a). This was particularly illustrated by the failure to isolate aerobic bacteria from a total of 341 of 551 (62%) transendoscopically collected tracheal wash samples in a study of IAD in young racehorses (Wood *et al.*, 1993a). Analysis of data from this study also showed a highly statistically significant decreasing trend with increasing inflammation score for the proportions of samples from which aerobic bacteria were not isolated (Table 1.2).

**Table 1.2: Linear trend for decreasing proportion of samples with increasing inflammation score from which aerobic bacteria were not isolated for data presented by Wood *et al.* (1993)**

| Inflammation score | Sterile tracheal washes | | Total tracheal washes examined | Odds Ratio* |
|---|---|---|---|---|
| | n | % | | |
| 0 | 155 | 76 | 205 | 1.00 (ref) |
| 1 | 120 | 69 | 174 | 0.72 |
| 2 | 42 | 51 | 83 | 0.38 |
| 3 | 24 | 27 | 89 | 0.12 |
| Total | 341 | 62 | 551 | |

$\chi^2$ for linear trend = 65.85 (P<0.00001)

*1.3.2.4 Cytological assessment of tracheal aspirates*

Methods for detailed cytological assessment of transendoscopically or transtracheally collected tracheal washes from horses have been described (Beech, 1975; Whitwell & Greet, 1984; Mair, 1987; Sweeney *et al.*, 1992; Freeman, 1997; Freeman & Roszel, 1997a; 1997b). As for assessment of tracheal mucus there have been various systems used to score airway inflammation based on indices of tracheal wash cytological parameters (Sweeney *et al.*, 1992), sometimes also incorporating a measure of visible tracheal mucus (Whitwell & Greet, 1984; Wood *et al.*, 1993a; Burrell *et al.*, 1996; Chapman *et al.*, 2000). Sweeney *et al.* (1992) classified tracheobronchial aspirates from racehorses according to neutrophils as a proportion of the cells seen in a representative area of aspirate smear, with the baseline 'normal' group having ≤20% neutrophils. Whitwell and Greet (1984) described a 3-point 'inflammation score' based on assigning a single point to

each of i) moderate or profuse tracheal mucus, ii) $\geq 10^3$ nucleated cells/ml of tracheal wash and iii) moderate or greater proportions of neutrophils in the tracheal wash. This system, with a minimum score of 0 and a maximum score of 3, has been used in a series of studies by the AHT (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood *et al.*, 1997b; Ward *et al.*, 1998) including the longitudinal study of respiratory disease in racehorses (Wood, 1999). This system was slightly modified to a 5-point score in a study of experimental ozone-induced pulmonary oxidant injury in horses (Mills *et al.*, 1996). Definitions for each grade of inflammation were prescriptive but included the possibility of increasing scores by 0.5 to 1 according to certain criteria, including bronchoalveolar lavage characteristics. The Whitwell & Greet (1984) system was also recently adapted as a 9-point score by Chapman *et al.* (2000) in a retrospective study of IAD in racehorses. Scores comprised the sum of 3 ordinal 0-3 scores for each of i) tracheal mucus, ii) cell count density and iii) neutrophil proportion as measured by percentage of cells on the smear. In their study Chapman *et al.* (2000) scored neutrophil proportions slightly differently to Sweeney *et al.* (1992), as those with <5% neutrophils were considered baseline 'normal' (score 0) and subsequent scores were assigned for 5-10% (score 1), 10-40% (score 2) and >40% neutrophils (score 3).

### 1.3.3 Bronchoalveolar lavage (BAL)

The use of bronchoalveolar lavage (BAL) to sample the distal airways of the equine lung has been used since the early 1980s and may be performed 'blindly' using appropriate catheter tubing or through a suitably long endoscope (>2 metres) that allows sufficient access to the narrower conducting airways (Fogarty, 1990; McGorum & Dixon, 1994; Moore, 1996). The relative merits of each of these BAL techniques have been discussed (McGorum & Dixon, 1994).

Results of cytological examination of aspirates from BAL and tracheal lavage sampling have been compared but with no clear agreement across studies as to whether or

not there is good correlation between the 2 techniques (Mair, 1987; Mair *et al.*, 1987; Derksen *et al.*, 1989; Winder *et al.*, 1991 Traub Dargatz *et al.*, 1992; Freeman *et al.*, 1993; Dixon *et al.*, 1995a). The relative merits of BAL and tracheal lavage have been debated with respect to i) their practicality in different types of horses (importantly among performance horses, endoscopic tracheal lavage does not require sedation), ii) their applicability in detecting focal pulmonary disease (BAL may miss focal disease whereas tracheal lavage samples all areas of the lung), iii) their relative clarity of cellular morphology (BAL generally has clearer cell morphology), iv) ease of differential cell counting (easier with BAL as less cellular clumping with mucus), v) degree of 'contamination' by degenerative cells, mucus and debris and subsequent ease of cytological interpretation (although BAL has less contamination, tracheal lavages are easily and accurately interpreted) (McGorum & Dixon, 1994; Dixon *et al.*, 1995a).

To this end BAL has tended to become the technique of choice in studies of allergic respiratory disease (Naylor *et al.*, 1992; McGorum & Dixon, 1993; McGorum *et al.*, 1993a; 1993b; 1993c; 1993d; Robinson *et al.*, 1996; Robinson, 2001). BAL is also frequently used with other ancillary tests in referred cases of respiratory disease (Dixon *et al.*, 1995a; 1995b; Dixon, 1997) and in EIPH pathogenesis research (D. Marlin – personal communication).

Tracheal lavage is used much more frequently in clinical investigations and routine monitoring of IAD in racehorses that are frequently subjected to repeated sampling of the respiratory tract (Whitwell & Greet, 1984; Burrell, 1985; Burrell *et al.*, 1986b; Wood *et al.*, 1993a; Burrell *et al.*, 1994; 1996; Wood *et al.*, 1997a; Christley *et al.*, 1999b; Wood, 1999; Chapman *et al.*, 2000; Christley *et al.*, 2001a; 2001b). This is because it is very well tolerated by both horse and trainer and importantly does not require sedation and the need for a subsequent withdrawal period from competition (McGorum & Dixon, 1994; Dixon *et al.*, 1995a).

In the studies presented in this thesis tracheal lavage and not BAL sampling was used for various reasons. Tracheal lavage was considered a more appropriate technique when repeated sampling of the airways was being conducted, particularly when these were only one week apart as for the Welsh Mountain ponies, because localised trauma and inflammation induced by the procedure is more likely with BAL. Endoscopic tracheal lavage is also better tolerated by horses (and trainers) compared to BAL and importantly does not require administration of sedatives, which, as prohibited substances under the rules of racing, may affect subsequent race entries. Tracheal lavage, particularly if conducted after exercise, effectively samples the whole of the lung and is therefore much less likely to miss focal disease in more cranial and ventral regions of the lung than BAL, which samples only a small dorsal and caudal area of the lung. Although, several authors claim that BAL produces superior quality cytology, the pathology laboratories at the AHT have many years' experience in the preparation and interpretation of high quality cytological smears from tracheal lavage samples and these have been shown to be just as useful as BALs.

## 1.4 Transferrin

### 1.4.1 Bacterial iron acquisition

Iron is both an essential requirement for bacterial cell growth and is toxic when present in sufficient concentration as free ions (Carter *et al.*,1995). Host mammalian environments maintain toxic free iron at very low concentrations that is inhibitory to bacterial growth through specific complex metabolic mechanisms (Nixon, 2000). Iron metabolism, involving interplay of absorption, transport and storage, is conducted by many different enzymes and proteins, which ordinarily maintains a bacteriostatic and bacteriocidal environment by preferential chelation of iron (Ganzoni & Puschmann, 1975; Bezkorovainy, 1981; Bullen, 1981; Ward *et al.*, 1996; Jurado, 1997).

The principal mammalian iron transport protein is transferrin, which binds iron strongly but reversibly, with iron being normally released within host cells via transferrin-receptor complexes following cellular endocytosis. However, some pathogenic bacteria have developed mechanisms for acquiring iron for growth from mammalian transferrin in these iron-restricted environments, by producing cell membrane-associated transferrin binding proteins (TBPs) that interact with transferrin and preferentially sequester iron (Cornelissen & Sparling, 1994b; Guerinot, 1994; Jurado, 1997; Ratledge & Dover, 2000). Bacteria that demonstrate such TBP iron acquisition include members of the *Neisseria* (Cornelissen & Sparling, 1994a), *Haemophilus* (Schryvers & Gray-Owen, 1992), *Pasteurella* (Lo, 2001; Ogunnariwo *et al.*, 1997) and *Actinobacillus* (Gerlach *et al.*, 1992) species, all of which are important pathogens of humans and domestic animals. Many pathogenic bacteria (such as *Yersinia, Vibrio* and *Pseudomonas* spp. and mycobacteria) use siderophore-mediated iron acquisition (reviewed by Ratledge & Dover, 2000). These organisms produce siderophores, which are iron binding agents, to preferentially chelate iron from some host molecules such as ferritin, transferrin and lactoferrin. However, as there is little evidence that the Streptococci or Pasteurellaceae use this mechanism to acquire iron, it will not be mentioned further here.

It has been conceived that immunisation of animals against bacterial TBPs may be an effective means of protecting against these infections (Finkelstein *et al.*, 1983; Jurado, 1997; Sparling *et al.*, 1994). Such a vaccination strategy has been shown to be effective for *Mannheimia haemolytica* in ruminants (Potter *et al.*, 1999) and inactivated vaccines with proven efficacy that incorporate *M. haemolytica* propagated under iron restricted conditions (so called iron regulated protein or IRP vaccines) are now commercially available. Similar technology is being used in the development of vaccines against *Actinobacillus pleuropneumoniae* in pigs (Goethe *et al.*, 2000; Van Overbeke *et al.*, 2001).

The restricted mammalian host ranges for some strains of TBP expressing bacterial species appear to be related to their ability to utilise only the transferrin of the specific host species (Schryvers & Gonzalez, 1990). Examples include *Haemophilus influenzae* (Schryvers & Gray-Owen, 1992) and *N. meningitidis* (Schryvers & Gonzalez, 1990) in humans, *H. somnus* in cattle (Yu *et al.*, 1992), *Pasteurella* spp. in ruminants (Ogunnariwo & Schryvers, 1990; Ogunnariwo *et al.*, 1991; Yu *et al.*, 1992) and *A. pleuropneumoniae* in pigs (Gonzalez *et al.*, 1990). This implies that there are important differences between transferrins of different mammalian species that preclude universal iron acquisition by bacteria with ability to express TBPs.

In Streptococci, an important class of pathogenic bacteria in both humans and animals, iron acquisition mechanisms in iron restricted conditions have not been thought to involve direct iron sequestration from transferrin by TBPs (Chhatwal *et al.*, 1985; Eichenbaum *et al.*, 1996;.Tai *et al.*, 1993). There is, however, evidence that lactoferrin, an iron binding protein closely related to transferrin, provides some innate immunity against infection with several streptococcal species by preferential chelation of iron. This has been demonstrated for *S. mutans* (Lassiter *et al.*, 1987; Arnold *et al.*, 1980; 1981) and *S. pyogenes* (Stenfors *et al.*, 2001) in man and *S. agalactiae* (Rainard, 1992; 1993) in mastitis in cattle. There is limited evidence that *S. pneumoniae* (Hakansson *et al.*, 2001; Hammerschmidt *et al.*, 1999) in man and *S. uberis* (Fang & Oliver, 1999) in mastitis in cattle may actually acquire iron from lactoferrin. Despite the lack of evidence for a direct role of transferrin in protection or predilection to infection by Streptococci and disease, there are some intriguing suggestions that iron saturation of transferrin may be important in the pathogenesis of streptococcal infections. In a study of prognostic factors for *S. pneumoniae* pneumonia in people, Lambert & Hunter (1990) identified that only 14% of patients with low levels of unsaturated transferrin and positive blood cultures survived compared to 80%-88% of people with negative blood culture and/or normal unbound iron

binding capacity. The authors suggest that insufficient unsaturated transferrin may have facilitated the bacteraemia and consequently contributed to fatal pneumococcal pneumonia. Brochu *et al.* (1998) demonstrated *in vitro* that *S. intermedius* was able to acquire iron in iron restricted conditions by causing its release from transferrin by rapidly decreasing the pH of the culture medium. They confirmed that this was not associated with TBPs or proteolytic activity and was prevented when the buffering capacity of the medium was increased.

### 1.4.2 Equine transferrin

Transferrin is one of the serum proteins that are frequently characterised according to their electrophoretic properties in the blood typing and parentage testing of horses (Jones & Bogart, 1971). Equine transferrin is a highly polymorphic trait with 14 alleles (referred to as haplotypes from here) currently recognised for the single locus of the transferrin gene (Bowling, 1986). As each horse has 2 alleles, one from each parent, this means that there are 105 potential combinations (phenotypes) of these haplotypes, with heterozygous animals having 2 transferrin types in their blood corresponding to each separate haplotype (Jones & Bogart, 1971). The frequency of transferrin haplotypes among different horse breeds has been characterised (Table 7, page 86 in Bowling [1986]) and shows that the most prevalent haplotypes in all breeds are transferrins D and F2.

Although the electrophoretic properties of equine transferrin are well recognised and utilised throughout the world for parentage testing and identification in horses, the relationship of different transferrin haplotypes and phenotype combinations with respect to differential release of iron to bacterial TBPs or through other mechanisms, and hence predisposing to bacterial disease, have not been reported in the horse. It is possible, however, that differences in electrophoretic properties between equine transferrin haplotypes might well be related to differences in competitive binding of iron (Charlwood &

Jarritt, 1974) in the presence of either TBP or other bacterial iron chelating mechanisms, resulting in different manifestations of infection and disease in horses possessing different haplotypes.

To this end, one aim of this thesis was to examine whether different transferrin haplotypes in Welsh Mountain pony foals were associated with differences in disease and infections of the respiratory tract. This would be presumed to involve infection with *Pasteurella* and *Actinobacillus* spp., which are known to be associated with equine inflammatory respiratory disease and are recognised as acquiring iron via TBPs. The background to how this came about is covered in the introduction to the study of naturally occurring respiratory disease in Welsh Mountain pony foals (Section 3; Chapter 6).

## 1.5 Streptococcus zooepidemicus

### 1.5.1 Association of *S. zooepidemicus* infection with equine respiratory disease

*Streptococcus zooepidemicus* is the bacterium most frequently isolated from cases of equine pneumonia and pleuropneumonia (Raphel & Beech, 1982; Welsh, 1984; Sweeney *et al.*, 1985b; Mair & Lane, 1989; Sweeney *et al.*, 1991; Chaffin & Carter, 1993) and has generally been regarded as the most important secondary pathogen of the respiratory tract of horses following primary viral infection (Gerber, 1970; Thomson, 1978; Allen & Bryans, 1986; Wood & Chanter, 1994; Allpress, 1997). Although most frequently associated with horses, diseases in other species including man may be associated with infection with *S. zooepidemicus* (Chanter, 1997). In man, unpastuerised milk from cows with mastitis may be the source of *S. zooepidemicus* associated sore-throats, septicaemia, meningitis and nephritis (Barnham *et al.*, 1983; Barnham *et al.*, 1987a; Barnham *et al.*, 1987b; Chanter, 1997). In addition, *S. zooepidemicus* may be associated with metritis and mastitis in cattle; arthritis, abortion and septicaemia in pigs; pleuritis, pericarditis and pneumonia in lambs; fatal septicaemia in chickens (Carter *et al.*, 1995) and mass mortality from pneumonia in

rodents such as coypu (Martino & Stanchi, 1998), guinea pigs (Weber & Bruner, 1979) and mice (Literak & Mraz, 1991).

Epidemiological studies have demonstrated significant associations between infection with *S. zooepidemicus* of the lower airways of racehorses and both IAD (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood, 1999; Chapman *et al.*, 2000) and coughing (Christley *et al.*, 2001b). These studies have also shown a strong association between these 2 syndromes and demonstrated that coughing is a specific but insensitive indicator of IAD (Burrell *et al.*, 1996; Christley *et al.*, 2001a) and that IAD associated with bacteria is not dependent on prior viral infection (Wood, 1999). However, to date there has been only one limited report of an experimental infection demonstrating that *S. zooepidemicus* can act as a primary pathogen (Varma *et al.*, 1984). The reasons for the current and foreseeable lack of evidence for *S. zooepidemicus* satisfying Koch's postulates of infectious causality relate to this being a ubiquitous infection of equids and the non-availability of specific-pathogen-free (SPF) foals to this organism. Consequently, early natural exposure of horses to *S. zooepidemicus* usually acts to confound experimental challenges (N. Chanter, unpublished observations). In addition, epidemiological studies have shown that whilst *S. zooepidemicus* infection may be the most important infectious factor in these disease syndromes, it is not absolutely exclusive. Other infections such as *Actinobacillus/Pasteurella* spp., *S. pneumoniae* (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood, 1999; Chapman *et al.*, 2000; Christley *et al.*, 2001b) and sometimes mycoplasmas and viruses may be involved in mixed infections (Wood, 1999). As an alternative to the 'gold standard' of experimental reproduction of disease with recovery of the organism from lesions, Wood & Chanter (1994) examined the criteria proposed by Hill (1965) for assessing whether the epidemiological association between IAD and *S. zooepidemicus* infection (in conjunction with *S. pneumoniae* and *Actinobacillus/Pasteurella* spp.) was likely to be causal. Re-assessing their arguments exclusively for *S. zooepidemicus*, Wood & Chanter (1994) were

able to satisfy at least 7 (strength of association, consistency, biological gradient, biological plausibility, coherence, some limited experimental evidence, analogy with other species) of the 9 criteria for causal association with IAD (Hill, 1965).

### 1.5.2 Subtyping of *S. zooepidemicus* isolates

Detection of *S. zooepidemicus* and its differentiation from other beta-haemolytic streptococcal species using standard bacteriological techniques is relatively straightforward (Cowan & Steel, 1993). The epidemiological studies described above have used these techniques in conjunction with standard quantification techniques (Wood *et al.*, 1993a; Wood, 1999) to identify and quantify *S. zooepidemicus* in tracheal washes and all analyses have considered this organism as an homogenous species. However, there have been several subtyping techniques described for isolates of *S. zooepidemicus* and these have used various technologies that have been available at different times. Such techniques provide the opportunity for more detailed molecular epidemiological investigations of the role of *S. zooepidemicus* in equine respiratory disease.

Moore & Bryans (1969) described the serotyping of equine isolates of Lancefield group C beta haemolytic bacteria by immunodiffusion precipitation using antiserum raised in rabbits inoculated with acid extracts of equine isolates of *S. zooepidemicus*. In the initial description of this method, 13 specific antigenic types were characterised and 68 of 164 (41%) equine isolates of *S. zooepidemicus* could be classified by this typing technique (Moore & Bryans, 1969)

Barnham *et al.* (1987a) described the characterisation of a panel of human (zoonotic) and animal isolates of *S. zooepidemicus* using various methods including the API20STREP biochemical typing method, antibiotic susceptibility, bacteriocin production and sensitivity and susceptibility to a panel of 14 group C streptococcal bacteriophages. Results using this range of different techniques generally demonstrated that types isolated

from the same outbreaks were similar and could be discriminated from other outbreak strains, particularly by bacteriocin production and sensitivity and bacteriophage susceptibility typing. However, there was also evidence that multiple strains were involved in some outbreaks of human disease. Subsequent 'DNA fingerprinting' of these same isolates using nuclease digestion and agarose gel electrophoresis confirmed many of these findings including large number of different types, the specificity of bacteriocin and bacteriophage *S. zooepidemicus* typing and that isolates from clusters of outbreaks did have identical 'prints' (Skjold *et al.*, 1987).

Other techniques such as random amplified polymorphic DNA analysis (RAPD), multilocus enzyme electrophoresis (MEE) and pulsed-field gel electrophoresis (PFGE) have also been applied to a restricted number of human and animal *S. zooepidemicus* isolates and have been found to discriminate different types (Bert *et al.*, 1996; Soedarmanto *et al.*, 1996; Bert *et al.*, 1997).

Jorm *et al.* (1994) used MEE to type 70 isolates of *S. equi* and 177 isolates of *S. zooepidemicus* recovered from horses on 2 Thoroughbred studs in New South Wales, Australia. Results confirmed that there was a large number of types (n=41) of varying prevalence identified by this method and that *S. equi* appeared as a single clone apparently derived from the antigenically more diverse archetypal *S. zooepidemicus* species.

Walker & Timoney (1998) investigated the molecular basis of variability in the M-proteins of *S. zooepidemicus* (SzP) isolates by sequencing and comparison of the M-protein genes of 14 of 15 different Moore and Bryans serovars. Two distinct M-protein types were identified on the basis of the amino acid sequences of the N-terminal corresponding to residues 27 to 48 and variability in the number of repeats of proline-glutamic acid-proline-lysine (PEPK) at the carboxy terminal. In addition, 5 distinct M-protein hypervariable regions (HV1-HV5) were identified corresponding to differences in amino acid sequences between residues 106 and 166. These typing techniques have also been used to demonstrate

that horses without clinical signs could simultaneously harbour different subtypes of *S. zooepidemicus* in their tonsils but that separate isolates from the lower airways of cases of pneumonia in donkeys and foals were identical and consistent with infection by a single clone (Timoney *et al.*, 1997; Anzai *et al.*, 2000). Further studies using typing by restriction fragment length polymorphism (RFLP) electrophoretic characteristics following digestion of SzP by the restriction enzyme *Dde I*, have shown at least 19 different *S. zooepidemicus* types among 194 isolates from 29 foals and 2-year-olds (Anzai *et al.*, 2001).

Chanter *et al.* (1997) used the 16S-23S RNA gene intergenic spacer to characterise different members of the Lancefield group C streptococci of *S. equi, S, zooepidemicus, S. equisimilis* and *S. dysgalactiae.* Nine distinct regions of the spacer were identified and variations in spacer regions 1, 3, 5, 6 and 7 were used to group all *S. zooepidemicus* and *S. equi* isolates into one of 8 possible intergenic spacer types (A1, A2, B1, B2, C1, C2, D1 and D2), with all *S. equi* being type D1 clones.

More recently Abdulmawjood & Lammler (2000) described using several differences in the V2 region of the 16S rRNA gene identified by RFLP using the *HincII* restriction enzyme and sequence comparison with *S. equi,* as well as variation in the size of the 16S-23S rRNA spacer gene to discriminate different strains of *S. zooepidemicus.*

In this thesis the 2 PCR typing methods based on characterisation of the M-protein hypervariable region (5 possible types) described by Walker & Timoney (1998) and the 16S-23S RNA gene intergenic spacer (8 possible types) reported by Chanter *et al.* (1997), were both applied to isolates of *S. zooepidemicus* from Welsh Mountain ponies suffering naturally occurring respiratory disease. Theoretically, the combination of these 2 typing systems would permit the allocation of each isolate to one of 40 (8×5) hypothetical types. Both typing techniques required similar PCR facilities and could be applied easily together under the same cycling conditions. The availability of published primer sequences meant

that this combined typing technique could be easily adopted by other laboratories and applied to *S. zooepidemicus* isolates recovered from any species, including man.

## 1.6 Design & analysis of studies of respiratory disease in horses

Although we believe that infections are important in the aetiology of equine respiratory disease, especially in young horses, to date the associations of bacterial infections with disease have not been universally accepted. In addition, data for modelling dynamics of specific infections, with the notable exception of equine influenza (Glass *et al.*, 2002), are not readily available. Therefore, methods for the specific modelling of infectious diseases have not been adopted in this thesis but are briefly mentioned here for completeness. These methods include *a posteriori* reasoning (Bregman & Langmuir, 1990) and various forms of *a priori* reasoning including the mass action concept (Fine, 1993; Anderson & May, 1991), Reed Frost models for acute contagious infections (Abbey, 1952; Fine, 1977) and complex multi-compartment models with deterministic or stochastic approaches (Anderson & May, 1985; 1991).

### 1.6.1 Cases series and outbreak investigations

Many studies of respiratory disease in horses have been either case reports/case series or descriptions of outbreaks and their investigation. Case reports and series tend to describe animals with specific clinical signs, series of signs (syndromes) or diagnoses that have usually been referred to particular centres over a period of time or seen by individual clinicians. Outbreak reports normally describe respiratory diseases occurring in a discrete group of horses, clustering in time and with a specific, single and usually infectious aetiology. There are many examples of these types of studies on equine respiratory disease among the literature and whereas they collate information from cases/outbreaks and provide useful descriptive information, they are usually limited by their lack of quantitative

assessment of specific risk factors for equine respiratory disease. Exceptions to this have been some outbreak investigations that have subjected data to simple quantitative analyses to examine particular risk factors (e.g. Wood *et al.*,1997a), particularly relating to the efficacy of respiratory vaccines (Jorm, 1990; Morley, 1995; Newton *et al.*, 1999a; 2000a).

### 1.6.2 Cases control studies

Case control studies have certain advantages and disadvantages compared with other types of study such as the cohort design (Schlesselman, 1982). On the positive side they are well suited to rare diseases and those with long latency, they are relatively quick and inexpensive to conduct, they require fewer individuals and frequently make use of existing data, they do not pose a risk to subjects and they allow multiple risk factors to be examined simultaneously. On the other hand they are frequently retrospective and as such rely on recall or on existing records and this may present problems with validation of data and control of confounding if data on potential confounders does not exist. In addition, there may be difficulties with control selection and rates of disease in exposed and non-exposed groups are not determinable (Schlesselman, 1982).

The aim of case control studies is to quantify the associations between diseases and exposures of interest (risk factors) within a given population. This is achieved i) by making comparisons with respect to frequency of exposures among appropriately selected diseased (cases) and non-diseased (controls) individuals and ii) because 'frequency measures of disease among the exposed' are logically related to 'frequency measures of exposure among the diseased' (Schlesselman, 1982).

The comparative measure (of either disease or exposure) underlying case control methodology is the 'odds ratio' (i.e. OR = 'odds of disease or exposure among cases'/'odds of disease or exposure among controls' with odds defined as 'number of diseased or exposed'/'number of non-diseased or non-exposed'). It can be shown mathematically,

assuming that the probability of cases and controls being selected is independent of their exposure status, that the 'exposure odds ratio' = ' disease odds ratio' (Schlesselman, 1982):

$$\underline{\text{Exposure Odds Ratio}} = \underline{\text{Disease Odds Ratio}}$$

$$\frac{\text{'odds of exposure among diseased'}}{\text{'odds of exposure among non-diseased'}} = \frac{\text{'odds of disease among exposed'}}{\text{'odds of disease among non-exposed'}}$$

Importantly also, because 'odds' = 'risk'/(1-'risk'), it follows that where the 'risk' of disease is small because it is rare in the population (e.g. 1 in 1000 diseased; 'risk' = 0.001), then the denominator for this equation is extremely close to 1 (i.e. 1-0.001 = 0.999) and consequently the 'odds of disease' closely approximate to the 'risk of disease'. In this situation the OR for a given exposure will also closely approximate to the risk ratio (RR) measure of relative risk between diseased and non-diseased individuals in the population.

| Risk of disease | | Odds of disease |
|---|---|---|
| risk = 1/1000 | | risk/(1-risk) |
| 0.001 | ≈ | 0.001/(1-0.001) = 0.001/0.999 |
| 0.001 | ≈ | 0.001001001… |

This is the basis for the rare disease assumption of case control studies, which generally states that for rare disease (prevalence<1%) the odds ratio, risk ratio (for prevalence measures) and rate ratio (for incidence measures) are, for all practical purposes, equal. However, more recently it has been shown that the rare disease assumption is not a prerequisite for case control studies because, with a suitable choice of sampling scheme for controls, it is possible to obtain direct estimates of risk and rate ratios instead of relying on the OR as an indirect estimate (Rodrigues & Kirkwood, 1990).

An important issue in case control studies is the use of 'matching', whereby controls are chosen from a subset of the available 'non-case' population with the subset of 'non-

cases' and the cases sharing a particular 'matching' variable. In human studies there is frequently use of age-matched controls i.e. the cases and controls come from a subset of the population that have the same age. The most important issues with respect to matching are that i) it may improve a study's precision, ii) it may help control confounding by factors that are difficult to measure (e.g. environmental factors), iii) it may be essential to overcome the rare disease assumption with more common disease (e.g. concurrent control selection described by Rodrigues & Kirkwood [1990]), iv) the effects of a matching variable cannot be evaluated in analyses, v) it makes cases and controls more similar and 'overmatching' may present problems, and vi) matched studies require statistical techniques that take appropriate account of the matching in analyses (e.g. conditional logistic regression).

Although case control studies are now widely used in human medicine and are increasing in popularity in veterinary medicine, there have been very few examples to date in the area of equine respiratory disease. Three main examples have been reported since 1993 (Hoffman *et al.*, 1993a; Austin *et al.*, 1995; Christley *et al.*, 1999b; 2001a; 2001b).

An unmatched case control study of risk factors for pleuropneumonia was conducted among horses referred to the veterinary teaching hospital at the Univeristy of Illinois, USA, between 1980 and 1990 in which 45 eligible cases were recruited (Austin *et al.*, 1995). Although there were no details provided for the population from which these cases arose, it may be reasonable to assume that with only 45 cases over 10 years that pleuropneumonia satisfied the rare disease assumption and the use of ordinary logistic regression analysis by these authors was valid (Austin *et al.*, 1995).

A small scale pilot study (8 cases and 8 controls) was conducted to establish a clinical definition of distal respiratory tract disease in foals on Thoroughbred breeding farms in Ontario, Canada (Hoffman *et al.*, 1993a). In this limited study the authors used the multiple matching criteria of farm, day of sampling, age, sex and weaning status and compared cases and controls for endoscopic, haematological and pulmonary cytological and

microbiological variables. Data were only presented for analyses at the univariable level by conventional statistical techniques and estimates of disease risk were not reported.

As already described, a matched case control study of coughing among racehorses at 5 racetracks in and around Sydney, Australia was conducted (Christley *et al.*, 1999b; 2001a; 2001b). In designing this study the authors were conscious of the need for careful choice of controls because of the likely violation of the rare disease assumption by this prevalent condition among young racehorses. The choice of a matched design with use of concurrently selected controls (i.e. temporal matching with cases eligible as controls later in the study) allowed the rare disease assumption to be overcome. Matching on training stable also helped control for other factors that were difficult to measure, including the sensitivity of observation by trainers, environmental conditions and some stable management and training factors (Christley *et al.*, 1999b). Having used a matched design, the appropriate technique of conditional logistic regression to take account of the matching on time and training stable was used to analyse the data (Christley *et al.*, 1999b; 2001a; 2001b).

### 1.6.3 Studies with repeated measures

It is important to state that this is not intended to be a comprehensive review of all the possible methodologies for dealing with the problems of repeated measures within all kinds of epidemiological studies. Rather it is a brief overview of the matters specifically relating to the nature of the data in a randomised, controlled trial in Welsh-Mountain ponies described later in this thesis.

Approaches to analyses of longitudinal data are comprehensively reviewed by Diggle *et al.* (2002) and may take three different forms of generalised linear models including marginal, random effects and transition models. In marginal models the correlation between outcome and explanatory covariates is taken as an average across individuals in the population that share common covariate values. In random effects models variation between

all individuals is modelled randomly and reflects natural heterogeneity due to unmeasured factors. Finally, in transition (or Markov) models outcomes are modelled in terms of explanatory covariates that include outcomes (and covariates) for the same individuals at previous time points. Although there are various recent examples of studies using these approaches, the modelling of bovine herpesvirus type 1 on herd-level milk production by Van Schaik *et al.* (1999) describes the application of all three modelling methods to the same data.

It is recognised that respiratory disease in horses, particularly in young animals with possible infectious aetiologies, clusters in both time and space (e.g. outbreaks of disease in training yards or groups of horses), and the infections change over time (Burrell *et al.*, 1996; Wood, 1999). Consequently it has been recognised that one of the most appropriate methods for collecting accurate information on the occurrence of respiratory disease in such groups of horses is by using a longitudinal study design (Wood *et al.*, 1993b; Burrell *et al.*, 1996; Wood *et al.*, 1997b; Wood, 1999). In longitudinal study designs individuals are followed over time with repeated observations made on them during the period of study. However, this does present specific issues with respect to the analysis of such data because the repeated measures effectively result in both clustering of data (i.e. observations) within individual animals and a consequent lack of independence between them.

Methods have been developed to account for such clustering of data in order to overcome the problems of i) artificially small standard errors for what may be otherwise accurate regression coefficient estimates but with consequently reduced P-values (Curtis *et al.*, 1988; Atwill *et al.*, 1995) or ii) inaccurate regression coefficients, reduced standard errors and P-values (Wood *et al.*, 1993b; Atwill *et al.*, 1995; Wood *et al.*, 1997b; Wood, 1999), both of which may arise from methods such as ordinary logistic regression that ignore clustering.

Mixed effect logistic regression (MELR) with random effects are useful because they can be applied to unbalanced data where there are observations on different individuals at different time points and with variable numbers of observations per individual (Hedeker & Gibbons, 1997; EGRET for Windows, 1999) and they provide some estimation of the strength of clustering (EGRET for Windows, 1999). MELR was used to analyse data from a longitudinal study of respiratory disease in young racehorses for the 3 binary outcomes of IAD, tracheal mucus and nasal discharge with random variation defined between (i.e. data were matched on) individual horses (Wood *et al.*, 1997b; Wood, 1999). It has been recommended that results of MELR should be presented retaining random effects terms even if these terms are not themselves statistically significant, as this provides the most accurate estimates for other effects having accounted for clustering of data (Mauritsen, 1984). In these analyses there were significant random effects for final MELR models for IAD and for tracheal mucus but not for nasal discharge, although results for this model were presented retaining a random effects term (Wood, 1999).

The MELR methods outlined above are available with various software packages including EGRET (EGRET for Windows, 1999) but although they are straightforward to apply they do have limitations. Such packages are only suited to binary outcome data from longitudinal/repeated measures studies and can only incorporate clustering at a single hierarchical level (e.g. observations within animals). However, there are also other statistical techniques and software available such as multilevel modelling in MLwiN (Multilevel Models project, Institute of Education), which are able to deal with either continuous or binary outcome variables and account for multiple hierarchical levels of clustering in data (Goldstein, 1999; Snijders & Bosker, 1999; Rasbash *et al.*, 2000).

Much of the text and software development on multilevel modelling has its basis in social rather than medical sciences and particularly within educational assessment (Aitken *et al.*, 1981; Aitken & Longford, 1986; Goldstein, 1999; Snijders & Bosker, 1999; Rasbash *et*

*al.*, 2000). However, because of the hierarchically structured nature of populations of most companion and particularly food producing species of animals (e.g. broilers within cages within sheds within farms), the considerable worth of multilevel modelling techniques are being increasingly recognised by veterinary epidemiologists as an appropriate means of dealing with complex patterns of variability within their data (e.g. Green & Morgan, 1993; Lancelot *et al.*, 1995; Kadohira *et al.*, 1997; Chriel *et al.*, 1999; Grohn *et al.*, 1999; Rattenborg *et al.*, 1999, Dohoo *et al.*, 2001; Wood *et al.*, 2001; Green *et al.*, 2002). Some of these studies used multilevel modelling to account for clustering in assessing risk factors and providing more accurate estimates of their effects (Green & Morgan, 1993; Lancelot *et al.*, 1995; Chriel *et al.*, 1999; Grohn *et al.*, 1999; Rattenborg *et al.*, 1999, Green *et al.*, 2002). Others use the technique to evaluate the relative contribution of different levels of the hierarchy to the total variability in the outcome of interest (Kadohira *et al.*, 1997; Dohoo *et al.*, 2001; Wood *et al.*, 2001). By doing this they are able to identify areas in which targeted interventions are likely to have the largest impact on disease or production.

To the author's knowledge multilevel modelling techniques of this nature have not previously been described for repeated measures on equine respiratory disease, although they were recently applied to assess the significance of different levels of clustering in fatalities of Thoroughbred horses on British racecourses (Wood *et al.*, 2001). One of the aims of this thesis was to explore data from a randomised, controlled trial of an experimental bacterial vaccine conducted in 29 Welsh Mountain ponies (Section 3). The techniques described were used to examine these data which comprised repeated clinical evaluations of signs of respiratory disease and results of microbiological sampling from the respiratory tract of all animals.

## 1.7 Context of this thesis

The work presented in this thesis is a continuation of the AHT's more recent investigations of equine respiratory disease. However, unlike much of the work by Burrell and Wood (referred to above), which concentrated on epidemiological characterisation of cases defined by largely subclinical IAD, this project concentrates mainly on the epidemiology relating to cases defined by clinically apparent respiratory disease. The change in case definition from earlier studies and subsequent comparison of factors associated with clinical and subclinical forms of respiratory disease, aims to determine whether these are different manifestations of the same condition or whether there are separate factors associated with each.

Studies were conducted in 2 distinct equine populations and used different study designs and analytical methods to examine the associations of infectious and non-infectious risk factors with clinical disease. There were some differences in the non-infectious factors examined as a consequence of fundamental differences in the 2 populations. The studies that form the basis of this thesis are i) a case control study of clinically apparent respiratory disease that was nested within the longitudinal study previously referred to, and ii) a randomised, controlled trial of an experimental bacterial vaccine that was conducted in recently weaned Welsh Mountain pony foals monitored for naturally occurring respiratory disease.

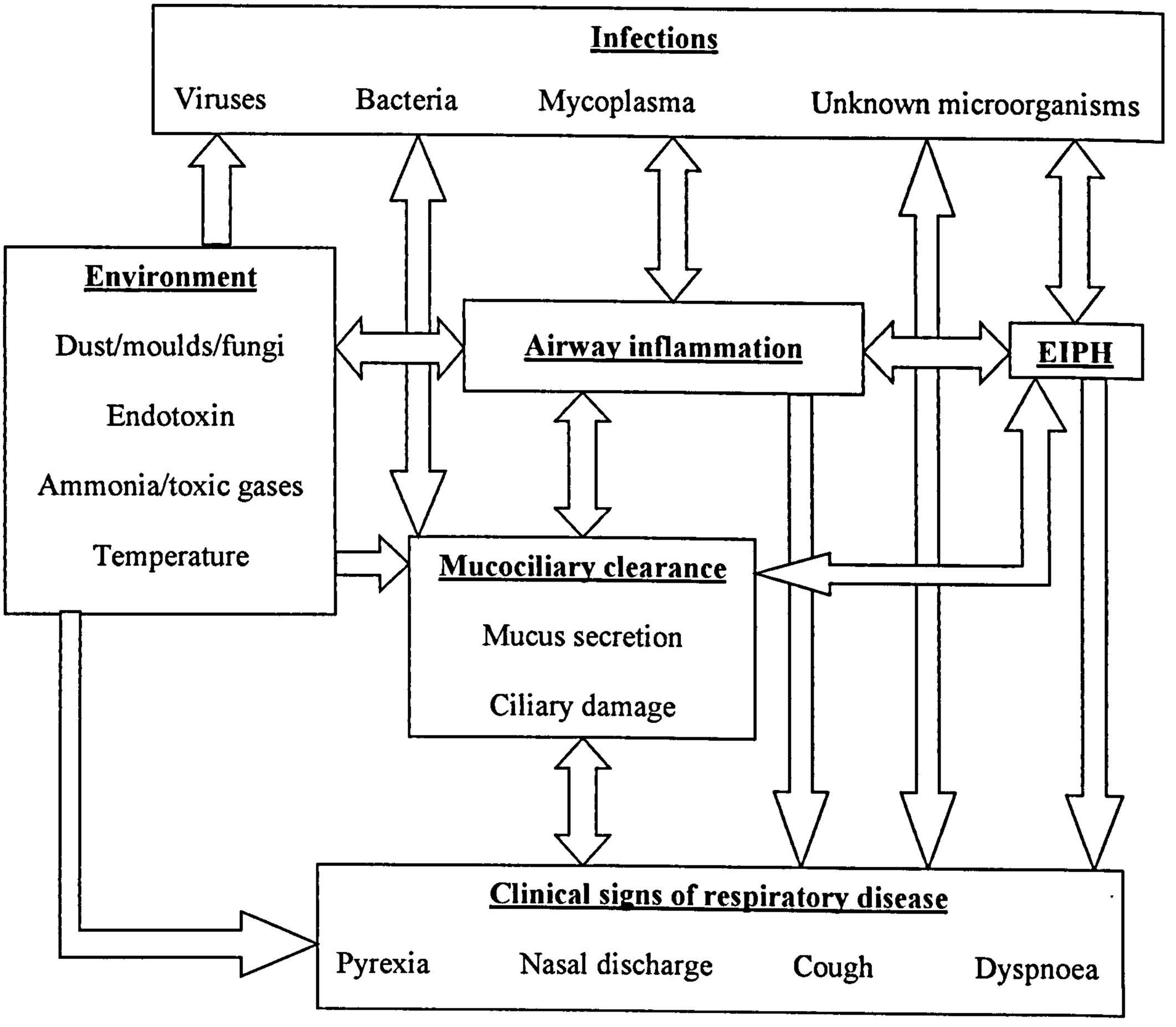
Appropriate case control methodology were used to investigate risk factors for clinically apparent respiratory disease in Thoroughbred racehorses, with data nested within a longitudinal study (Wood, 1999). The study differs from that reported in Australian racehorses (Christley *et al.*, 1999b; 2001a; 2001b) because analyses were conducted to gain an insight into the similarities in risk factors associated with clinical and subclinical presentations of respiratory disease.

The 2 PCR typing methods based on characterisation of the M-protein hypervariable region (5 possible types) described by Walker & Timoney (1998) and the 16S-23S RNA gene intergenic spacer (8 possible types) reported by Chanter *et al.* (1997), were both applied to isolates of *S. zooepidemicus* from Welsh Mountain ponies suffering naturally occurring respiratory disease. Theoretically, the combination of these 2 typing systems would permit the allocation of each isolate to one of 40 (8×5) hypothetical types. Both typing techniques required similar PCR facilities and could be applied easily together under the same cycling conditions. The availability of published primer sequences meant that this combined typing technique could be easily adopted by other laboratories and applied to *S. zooepidemicus* isolates recovered from any species, including man.

### 1.7.1 A causal web for equine respiratory disease

Figure 1.8 summarises a possible causal web for equine respiratory disease which attempts to bring together many different but inevitably related factors that may be contributing to different subclinical as well as clinical presentations of respiratory disease in the horse.

**Figure 1.8: A causal web for equine respiratory disease**



# SECTION 2

# A CASE CONTROL STUDY OF CLINICALLY APPARENT RESPIRATORY DISEASE IN THOROUGHBRED RACEHORSES

# CHAPTER 2

# INTRODUCTION

## 2.1 Background

During the 1980s and early 1990s it was increasingly recognised that respiratory disease was a common and recurrent problem in young Thoroughbred racehorses and that whilst this may be clinically obvious it was more frequently subclinical, being characterised by the presence of airway mucus and inflammatory exudate (Burrell *et al.*, 1986; 1996). In order to estimate for the first time the incidence and prevalence of both clinical and sub-clinical forms of respiratory disease and the association between disease and various factors, including infections by different pathogens, a longitudinal study comprising repeated and regular sampling of individual Thoroughbred racehorses was conducted (Wood, 1999).

Sampling and data collection for the study took place between November 1993 and December 1996 and involved a total of 148 horses in 6 different training yards with horses from only 5 yards being studied at any one time. For practical reasons and to ensure maximum co-operation from trainers and their staff for the duration of the study, a representative sample of between 10 and 15 horses in each yard were examined and sampled on a monthly basis irrespective of the respiratory health status of horses.

This longitudinal study of respiratory disease in Thoroughbreds was funded by a grant awarded to the principal investigator, James Wood, by the Horserace Betting Levy Board. The majority of monthly examinations and sampling of horses was conducted by myself and I was responsible for collating and reporting of all results to racehorse trainers and their veterinary surgeons on a monthly basis. Bacterial and cytological examinations of tracheal wash samples and serological testing of blood samples were all conducted in the microbiology and pathology laboratories of the AHT. A commercial mycoplasma laboratory (Mycoplasma Experience) conducted examination of tracheal wash samples for

mycoplasmas. The longitudinal study was the basis of James Wood's PhD thesis, entitled 'An Epidemiological Investigation of Respiratory Disease in Racehorses' (Wood, 1999), which is referenced in these methods and elsewhere in this thesis. The case control study of clinically apparent respiratory disease described here, although nested within the longitudinal study, was planned and conducted by myself using a separate study design with a completely different case definition to that used in James Wood's analyses.

## 2.2 Aims of the case control study

The main aim of the study was to describe, quantify and test the significance of the associations of different infectious agents and other predisposing factors with clinically apparent respiratory disease in young Thoroughbred racehorses in training in the United Kingdom.

During the study it was recognised that endoscopic examination of some outwardly healthy horses revealed the presence of subclinical IAD. Therefore, classification of horses already selected as outwardly healthy controls as either 'healthy' or 'subclinical' would allow additional investigation and comparison of factors associated with subclinical as well as clinically apparent respiratory disease. This would allow evaluation of whether these were likely to be separate disease entities with different aetiologies or merely the same disease manifested to different extents.

More specifically the study aimed to determine whether both clinically apparent and subclinical respiratory diseases in young racehorses in training in the United Kingdom were associated with:

i) infection of the lower respiratory tract (LRT) by certain species of bacteria (*S. zooepidemicus, S. pneumoniae*, *Pasteurella* and *Actinobacillus* spp. particularly) including *Mycoplasma* spp. (*Mycoplasma felis* and *Mycoplasma equirhinis* particularly)

ii) isolation of species of bacteria from the upper respiratory tract (URT) that are associated with LRT disease,

iii) infection with known equine viruses,

iv) horses' age,

v) time since entry into the training yard,

vi) recent racing.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Study design

A matched case control study design with concurrent selection of controls (Rodrigues & Kirkwood, 1990) was used. This case control study was nested within a longitudinal study of respiratory disease (Wood, 1999). In addition to cases of clinically apparent respiratory disease in routinely monitored horses, other horses with clinical respiratory signs were also investigated during the study period if requested by the trainer. Controls were selected from horses that were routinely monitored in the study yards close to the time of the case and which did not have clinical signs of respiratory disease.

### 3.1.1 Longitudinal study of respiratory disease in Thoroughbred racehorses

*3.1.1.1 Racing yards*

A convenience sample of 6 Thoroughbred training yards was used for the study, with horses from only 5 yards being studied at any one time. A convenience rather than random sample of trainers was used in order to ensure that there would be a high degree of co-operation from the yards for a considerable part of the 3 years of the study, thus ensuring the highest possible quality of repeated measures data. Yards were, therefore, recruited on the basis of likely good co-operation based on known previous interest in equine respiratory disease from both the trainers and their veterinary surgeons, although before recruitment there was no reason to believe that these yards suffered any worse than others with respiratory disease.

Of the 6 training yards involved in the study, 4 were situated in Newmarket in Suffolk and one each in Epsom in Surrey and Lambourn in Berkshire. Although 6 training yards were involved in the study, a total of 7 trainers provided data for their horses. This

was because of the untimely death of trainer 7 who was replaced on the study by his successor at the yard (trainer 5) . A summary of details of the location, size, age structure and aspects of construction and management of the different yards under each trainer is outlined in Table 3.1, (adapted from Wood [1999]).

**Table 3.1: Details of the training yards studied in the longitudinal study (adapted from Wood [1999])**

| | Trainer 1 | Trainer 2 | Trainer 3 | Trainer 4 | Trainer 5 | Trainer 6 | Trainer 7 |
|---|---|---|---|---|---|---|---|
| **Location** | Lambourn | Newmarket | Newmarket | Epsom | Newmarket | Newmarket | Newmarket |
| **Horses (approx. n)** | 55-65 | 100-125 | 65 | 55-70 | 70 | 175 | 90 |
| **Period studied** | 14 months | 34 months | 36 months | 36 months | 27 months | 12 months | 6 months |
| **% 2 year olds** | 55% | 58% | 55% | 15% | 60% | 45% | 55% |
| **% 3 year olds** | 35% | 36% | 35% | 30% | 35% | 50% | 30% |
| **% ≥4 year olds** | 10% | 6% | 10% | 55% | 5% | 5% | 15% |
| **Type of yard** | Flat only | Flat only | Flat only | NH & Flat | Flat only | Flat only | Flat only |
| **Construction** | Loose boxes | American barns & loose boxes | Loose boxes | Loose boxes | American barns | American barns & loose boxes | American barns |
| **Bedding type** | Shavings | Paper/shavings | Straw | Straw | Shavings | Shavings | Paper/shavings |
| **Shared airspace** | No | Yes | Yes & No | No | No | Yes & No | No |

### *3.1.1.2 Thoroughbred racehorses*

At the time of recruitment to the study, trainers were asked to select at random between 10 and 15 Thoroughbred racehorses that were representative of the age, sex and general management structure within each yard and which were not intended to be sold midway through the year. Trainers were specifically requested not to select horses on the basis of their previous respiratory disease and horses that were known to be temperamentally difficult with respect to respiratory sampling were excluded on the grounds of safety. Horses that were sold or left the yard for prolonged periods during the year were replaced by another horse.

### *3.1.1.3 Routine monthly examination and sampling*

Simple clinical examination was performed on horses at the time of the monthly visit to the training yard immediately prior to samples being collected. The presence or absence of abnormal and obvious serous or mucopurulent nasal discharge and coughing was noted.

A blood sample was then collected from the jugular vein into a silicon coated evacuated blood collection tube (Vacutainer™; Becton-Dickinson). After the blood had clotted, serum was poured off and used for viral serological testing.

An unguarded wire-mounted gauze swab was passed via the ventral nasal meatus to the level of the common pharynx and soft palate. The swab was then gently moved around several times to slightly abrade the mucosa before being removed. The gauze swab was immediately placed and sealed in transport medium (isotonic phosphate buffered saline (PBS), pH 7.2, containing 2% (v/v) foetal calf serum and 0.0005% (w/v) amphotericin B (Sigma-Aldrich) (Newton *et al.*, 1997b). Nasopharyngeal swabs were transported to the AHT laboratories on ice blocks in polystyrene boxes and were submitted for bacteriological examination.

The respiratory tracts of the horses were then evaluated for the amount of mucus and/or blood visible in the trachea and this was recorded as either absent (score = 0), slight (score = 1), moderate (score = 2) or severe (score = 3), as illustrated in Figure 3.1 (from Wood [1999]). Examination took place to the level of the tracheal bifurcation (carina) using a flexible 1.0m-1.7m endoscope (various makes used) which was passed rapidly but carefully through the ventral nasal meatus, nasopharynx, larynx and then into the trachea.

**Figure 3.1: Tracheal mucus scoring (from Wood [1999])**

(a) (b) (c)

**Tracheal mucus scores, from left: (a) 1/3 (b) 2/3 and (c) 3/3. The degree of blood or haemorrhage was scored on the same scale.**

Tracheal wash samples were collected transendoscopically by the instillation and aspiration of 30ml of sterile phosphate buffered saline (PBS) just anterior to the bifurcation using a flexible, sterile polythene catheter passed through the biopsy channel of the endoscope (Greet, 1982; Whitwell & Greet, 1984; Dixon *et al.*, 1995b). Immediately following collection and thorough mixing, tracheal wash samples were divided into 3 aliquots. One aliquot went into 15% (v/v) ethylene diamine tetra-acetic acid (EDTA) for nucleated and red blood cell counts, another was fixed with an equal volume of 10% (v/v) saline buffered formalin for cytological examination and the remaining neat sample was transported to the laboratory on ice blocks for quantitative bacteriological examination.

After each examination the endoscope was wiped clean and disinfected by immersion in liquid disinfectant (10% (v/v) TriGene™, MediChem International, Sevenoaks, Kent, UK), with particular care taken to make sure that the biopsy channel was cleaned. Following disinfection the endoscope, including the biopsy channel, was thoroughly rinsed using sterile saline.

*3.1.1.4 Examination and sampling of other horses*

In addition to the routine monthly sampling of horses selected by trainers for the longitudinal study, which was conducted irrespective of the presence of respiratory signs, other horses in the yards that showed signs of clinically obvious respiratory disease were also examined. Horses that had sudden onset coughing, an obvious, abnormal nasal discharge or a high rectal temperature (≥38.6°C/101.5°F) were examined when requested by the trainer. These additional examinations were conducted both separately from and at the same time as the routine monthly yard visits.

### 3.1.2 Sample size calculations

As this study was nested within the 3 year longitudinal study of inflammatory respiratory disease (Wood, 1999) it was, therefore, largely reliant on the sampling conducted for that study. No formal sample size calculations were performed prior to the collation of data for this case control study. After collating case data from within the completed longitudinal study, sample size calculations were performed *post hoc* using standard epidemiological methods for matched case control studies (Breslow & Day, 1980) in order to estimate the likely strengths of effect that might be required for a given level of significance and power. It was estimated that using a case:control ratio of 1:4, and an 80% chance (power) of detecting the effects of an exposure with 30% prevalence and an OR of 2.0 with 95% confidence, 74 matched case control sets were required. However, as we had many more cases than matched sets this was likely to be a conservative estimate of the expected significant OR at this exposure level. However, estimates of sample size requirements for matched case control studies are imprecise because they are profoundly affected by the closeness of matching and hence the correlation between exposure status of matched cases and controls (Dupont, 1988).

### 3.1.3 Matching

Matching was used in this case control study to overcome several potential confounders. All cases and controls with the same matching criteria were treated as a matched set for purposes of analysis. All cases of clinically apparent respiratory disease were matched to controls:

i) by trainer (and hence training yard as no trainers moved yards during the course of the longitudinal study) and

ii) to within a period of 6 weeks of sampling of the case and other controls in the set.

Matching by trainer controlled for factors that were unique to each training yard and trainer and which were difficult to measure and adjust for by other methods such as stratification or regression. Such factors included those related to the general management of horses, including housing (and hence ventilation, bedding etc.), feeding, daily routine, training regime, contact with other horses and hence their infections (clustering in space) and likelihood of receiving veterinary investigation and treatment and being notified as a case.

Matching of cases and controls to within a 6 week period satisfied the requirement for concurrent control selection in the use of a case control study design (Rodrigues & Kirkwood, 1990). A period of 6 weeks (approximately 1.5 times the routine sampling interval) for time matching cases and controls was used pragmatically to allow maximum recruitment of controls as cases were not necessarily sampled at the same time as the routine sampling in the longitudinal study. A shorter period was considered to prohibitively restrict the number of matched sets. Matching on time also controlled the effects of season and related factors such as the variable introduction of new horses and variations in intensity of training and racing. An important consideration in the study of respiratory disease of probable infectious aetiology among horses is the possible confounding by clustering in time. Matching on time was designed to control this.

Matching was restricted to trainer and time period to avoid the risk of overmatching. There was no need to match on covariates such as age as these could be measured and their effect estimated.

## 3.2 Outcome variable: definition and selection of cases

Cases of clinically apparent respiratory disease were defined as all horses that were resident in study yards during the period of the longitudinal study, which on examination demonstrated at least one of the following signs:

i) sudden onset coughing at exercise or rest

ii) abnormal and obvious serous or mucopurulent nasal discharge

iii) high rectal temperature/pyrexia (≥38.6°C [101.5°F]).

Clinically apparent cases of respiratory disease were identified irrespective of endoscopic findings or cytological interpretation of tracheal washes. Some cases were being routinely monitored and others were presented by the trainer for examination.

## 3.3 Outcome variable: definition and selection of controls

### 3.3.1 Selection of 'all' controls

Where possible and subject to restrictions required for matching, up to 4 matched controls were initially selected at random 'out of a hat' for each case from eligible horses routinely sampled during the longitudinal study. Horses were eligible as controls if i) they did not have clinical signs of respiratory disease (coughing, nasal discharge or pyrexia) at the time of sampling, ii) they were under the same trainer and iii) they had been sampled within 6 weeks of examination of the case. This selection was made irrespective of endoscopic findings or cytological interpretation of tracheal washes. All sets of cases and controls matched by trainer and time period were assigned a unique identifying number (set matching). The binary variable CASE took the value of 'one' for clinically apparent cases and 'zero' for 'all' controls.

### 3.3.2 Subdivision of controls

*3.3.2.1 Inflammation score*

A summary measure of airway inflammation was made using an aggregated scoring system comprising 3 equally weighted parameters (Whitwell & Greet, 1984). A score of one was given for each of:

i) moderate or severe amounts of endoscopically visible tracheal mucopus

ii) ≥1000 nucleated cells/ml of tracheal wash

iii) moderate or predominant amounts of neutrophils in the tracheal wash.

Under this scoring system the degree of inflammation ranged from zero to a maximum score of 3, with a score of zero indicating no evidence of airway inflammation. The detailed definition of each component is given with the description of the laboratory methods (see 3.4 Laboratory methods).

*3.3.2.2 Definition and selection of 'healthy' controls*

Control horses were defined as 'healthy' if endoscopic findings and tracheal wash cytological parameters demonstrated no evidence of lower respiratory tract disease, i.e. as indicated by an inflammation score of zero out of a possible maximum score of 3. The binary variable CASE2 took the value of one for clinically apparent cases and of zero for 'healthy' controls (subclinical controls were classified as 'missing' values) and variable CASE4 took the value of one for 'subclinical' controls (dealt with as 'cases' in this comparison) and of zero for 'healthy' controls.

*3.3.2.3 Definition and selection of 'subclinical' controls*

Control horses were defined as having 'subclinical' respiratory disease if endoscopic findings and tracheal wash cytological parameters demonstrated evidence of airway inflammation, as indicated by an inflammation score of one or more (Table 3.2). The binary variable CASE3 took the value of one for clinically apparent cases and of zero for 'subclinical' controls (healthy controls were classified as 'missing' values) and variable CASE4 took the value of one for 'subclinical' controls (dealt with as 'cases' in this comparison) and of zero for 'healthy' controls.

**Table 3.2: Summary of inflammation scores used to define the 2 control classifications**

| Control Classification | Inflammation score | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| Healthy | + | – | – | – |
| Subclinical | – | + | + | + |

+ score used in control selection
– score used in control exclusion

## 3.4 Laboratory methods

### 3.4.1 Cytology

Cytological examinations of tracheal wash samples, including nucleated and red blood cell counts, were conducted in the AHT laboratories by appropriately trained technicians and pathologists according to standard methods by Whitwell and Greet (1984).

Samples for cell counts were submitted in potassium EDTA tubes and where specimens were visibly mucoid, trypsin was added to break down mucus. A Neubauer counting chamber was used to separately count nucleated and red blood cells. Cell counts were expressed as the number of cells per ml of wash.

Samples for cytological examination were submitted mixed in equal volumes with 10% formal buffered saline as a fixative. The samples' visual characteristics of clarity and flocculation were recorded. Trypsin was added to markedly mucoid specimens. Samples were then centrifuged at 1200 rpm for 5 minutes to sediment the cells. All but 1ml of the supernatant fluid was then discarded. The pellet was re-suspended with the remaining 1ml of supernatant and 1-2 drops were smeared onto 2 gelatinised slides. The smears were fixed with polyethylene glycol, one was stained with haematoxylin and eosin (H&E) stain and the other with Perle's stain prior to examination under the microscope.

The fixed and stained tracheal wash smears were examined for the presence of inflammatory and degenerative changes using the techniques described by Whitwell and

Greet (1984). Records were made of the smear cell density, mucus quantity and characteristics, relative proportions of different cell types including haemosiderophages (i.e. alveolar macrophages containing Perle-positive, erythrocyte derived haemosiderin deposits) as well as other endogenous and exogenous material. Categories of recorded cytological characteristics are summarised in Table 3.3.

**Table 3.3: Summary of cytological variable categories**

| Cytological variable | Categories |
|---|---|
| Appearance of tracheal wash | clear, translucent, opaque |
| Flocculation of tracheal wash | slight, moderate, heavy |
| Nucleated & red blood cell counts | cells/ml of tracheal wash |
| Smear cell density | low, medium-low, medium, medium-high, high |
| Cell types: | |
| Neutrophils, macrophages, | +++ predominant cell type |
| Mononuclear cells, epithelial cells | ++ present in moderate proportions |
| & eosinophils | + small proportion of cells |
| | ± occasional cells only |
| | - no cells seen |
| Haemosiderophages | relative quantity on smear measured as +, ++, +++ or ++++ |
| Multinucleated macrophages | presence noted or not |
| Foamy macrophages | presence noted or not |
| Mucus quantity | scant, copious |
| Mucus characteristics | fine, inspissated |
| Plant material | presence noted or not |
| Fungal material | presence noted or not |
| Bacteria | presence noted or not |
| Filamentous structures | presence noted or not |
| Debris | presence noted or not |
| Degenerate epithelium | presence noted or not |
| Free ciliary tufts | presence noted or not |
| Squames | none, small or large quantity noted |
| Inflammation score (out of 3) | 0, 1, 2, 3 |

A summary measure of airway inflammation was made using an aggregated scoring system comprising 3 equally weighted parameters. A score of one was given for each of:

i) moderate or severe endoscopically visible tracheal mucopus,

ii) ≥1000 nucleated cells/ml of tracheal wash and

iii) moderate or predominant amounts of neutrophils in the tracheal wash.

Under this scoring system the degree of inflammation ranged from zero to a maximum score of 3, with a score of zero indicating no evidence of airway inflammation.

### 3.4.2 Serology

Serological tests were conducted by trained and experienced AHT laboratory technicians according to established standard operating procedures (Thomson *et al.*, 1976; Powell *et al.*, 1978). Blood samples were collected into silicon-coated plain tubes and after clotting and centrifugation serum was collected into a separate tube and stored frozen at –20°C. At the end of the data collection phase of the study all samples for each horse were batch tested in parallel to quantify antibodies to equine respiratory viruses. Samples were tested for equine herpesvirus-1 (EHV-1), equine herpesvirus-4 (EHV-4) and equine rhinovirus-1 (ERV-1) and equine rhinovirus-1 (ERV-2) by complement fixation (CF) test. Samples were also tested for equine adenovirus, the recent European-like equine influenza virus strain A/Newmarket2/93 (H3N8) and the prototype A/Prague/56 (H7N7) strain by haemagglutination inhibition (HI) test.

As repeated samples had been taken at monthly intervals from horses in the study, examination of rises in antibody titres between consecutive months would indicate likely infection (or vaccination in the case of influenza virus) around the time of the first of the 2 samples. A 4-fold or greater rise in antibody titres between consecutive monthly samples was used to determine serological evidence of infection for all viruses other than influenza. In the case of influenza virus, a 4-fold or greater rise in both the H7N7 and H3N8 subtypes of virus indicated probable vaccination around the time of the first sample. As H7N7 subrtypes were included in vaccines used at the time and had not been shown to be the cause of infection in horses anywhere in the world since the early 1980s, a 4-fold or greater rise in titre for the H3N8 subtype only, in the absence of recent vaccination was indicative of infection with the virus at around the time of the first sample.

### *3.4.2.1 Complement fixation (CF) test*

Complement fixation, based on established methods for equine sera (Thomson *et al.* 1976), was used to measure antibodies to EHV-1, EHV-4, ERV-1 and ERV-2. Sera were diluted 1 in 5 with CF diluent (5 complement fixation tablets (ICN Flow) per 500 ml distilled water) and inactivated in a waterbath for 30 minutes at 60°C. Further 2-fold serial dilutions of these diluted sera were made across 8 wells of a 96-well microtitre plate. Appropriately diluted antigens were added to each well in equal volume (25 µl) to the diluted serum and the plates were cooled to 4°C for ≥30 minutes. Freshly thawed Guinea-pig complement (25 µl) was then added to each well and the plates were stored overnight at 4°C. Fresh, washed sheep red blood cells diluted to a 4% (v/v) concentration in CF diluent and mixed with an equal volume of rabbit haemolysin solution (Serotec Ltd, Oxford, UK) were also stored at 4°C overnight. The red blood cells were then sensitised the following morning by warming to 37°C for 30 minutes and a 25µl aliquot was then added to each well, the plates were shaken and re-incubated at 37°C for 15 minutes twice. The plates were then shaken a third time and placed in the fridge at 37°C for 2-4 hours and read by visually assessing the degree of lysis of the red blood cells in each well and hence for each serum dilution. Controls for complement, all antigens and corresponding antiserum were used in every test and results outside acceptable limits were discarded. Anti-complementary activity (i.e. non-specific fixation of complement giving rise to a false positive result) was assessed for each sera by performing the tests in the absence of antigen.

If virus-specific antibody is present in serum, when antigen is added an immune-complex forms. Complement is then added and is fixed by adsorption to the immune-complex. When sensitised sheep red blood cells are added they will not be lysed due to the lack of free complement. In the absence of virus-specific antibody in the serum, immune-complexes will not form, complement is not fixed and is available to lyse the red blood cells.

*3.4.2.2 Haemagglutination inhibition (HI) test*

Antibodies to equine influenza virus and equine adenovirus were measured by haemagglutination inhibition, based on established methods for equine sera (Powell *et al.*, 1978). Aliquots of serum samples were diluted serially 2-fold across a 96 well microtitre plate in phosphate buffered saline (PBS). A standard virus antigen dose of 4 haemagglutination units (HAU) was then added to each well and the plates were incubated at room temperature for 30 minutes. A 1% (v/v) suspension of chicken red blood cells in PBS for the influenza HI test and a 1% (v/v) suspension of human O red blood cells for the adenovirus HI test were added to each well and the plates were shaken. After 45 minutes at room temperature the plates were read. Red blood cells cross-linked by virus haemagglutinin are unable to sediment and form a button of cells in the bottom of the V-bottomed well. Antibody is detected by absence of agglutination due to inhibition of cross-linkage and consequent formation of a clearly visible button in the bottom of the well. The end point titre was the highest dilution of serum that gave a >90% inhibition of haemagglutination in the well. Antigen and antiserum controls were tested on each plate and results discarded if the virus back-titration was not standard or if the control antiserum was not within a 2-fold dilution of that expected.

### 3.4.3 Bacteriology

Bacteriological assays were conducted by AHT laboratory staff according to standard operating procedures on non-fixed tracheal wash samples and nasopharyngeal swabs stored and transported in transport medium.

After vortexing, tracheal washes were diluted serially in PBS to produce subsamples diluted to $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$. Volumes of 0.1ml of each dilution were aseptically spread on various types of solid bacteriological media, including Wilkins-Chalgren 5% (v/v) horse blood agar (Oxoid), selective streptococcal agar (Strepselect; Mast Diagnostics),

MacConkey agar (Oxoid) and Wilkins-Chalgren 10% (v/v) horse blood agar and neomycin (Oxoid). All 4 agar types were used to inoculate samples neat and diluted $10^{-3}$. Horse blood and MacConkey agars were inoculated with samples diluted $10^{-1}$ and Strepselect was inoculated with samples diluted $10^{-2}$ and $10^{-4}$.

Primary inoculations of nasopharyngeal swabs onto Strepselect, horse blood, cystine lactose electrolyte deficient (CLED: Oxoid) and G20 agar (Oxoid) plates for the selective culture of *Bordetella bronchiseptica* (Smith & Baskerville, 1979), were spread with a sterile loop to obtain single colonies of bacteria after incubation.

Horse blood, Strepselect and MacConkey agars were all incubated overnight at 37°C. Horse blood and Strepselect were incubated in an atmosphere of 5% (v/v) $CO_2$. Neomycin blood agar plates were incubated anaerobically at 37°C for 4/5 days after the addition of a metronidazole sensitivity disc to the primary part of the swab inoculation.

Following incubation, plates of diluted tracheal wash samples were examined and separate counts were made of colonies of the different types of bacteria that could be presumptively identified by the type of media they grew on and by their colony morphology. The plates inoculated with a sample dilution with between 30 and 330 colonies were used for counting. The numbers of each species present were estimated and expressed as colony forming units per ml (cfu/ml) of neat tracheal wash sample.

Species of bacteria were identified using standard bacteriological techniques (Cowan & Steel, 1993). *Actinobacillus/Pasteurella* spp. were presumptively identified on the basis of reactions in the API 2ONE test strip identification system (BioMerieux). Those growing on MacConkey agar with a bi-polar staining, pleomorphic, Gram-negative cellular morphology and whose colony growth was sticky to the touch were presumptively identified as *Actinobacillus equuli*. Colonies with the same general morphology that did not grow on MacConkey agar and were not sticky were referred to as miscellaneous *Actinobacillus/Pasteurella* spp.

### 3.4.4 Mycoplasmology

*Mycoplasma* spp. were isolated from tracheal wash samples by the laboratories of Mycoplasma Experience Ltd in Reigate, Surrey. Tracheal wash samples from yard 4 in Epsom were generally delivered directly to the laboratory shortly after collection. Aliquots of washes from horses in the other yards had foetal calf serum added to a concentration of 10% (v/v) and samples were then stored frozen at -70°C. Batches of stored samples were periodically couriered overnight to Mycoplasma Experience on ice blocks in sealed, insulated polystyrene boxes.

On arrival at Mycoplasma Experience samples were thawed to room temperature and then 0.1ml of each wash was inoculated on ME Mycoplasma agar plates, which were then incubated in 95% (v/v) $N_2$ and 5% (v/v) $CO_2$ for up to 28 days. Plates were inspected microscopically at least twice weekly during the first 4teen days and again at 28 days. Media batches were validated for their ability to grow low numbers of pre-counted, frozen suspensions of a low-passage equine isolate of *M. felis*. Isolates with variations in colony morphology were sub-cultured.

Isolates were grouped according to their growth characteristics, their ability to metabolise arginine and ferment glucose in ME liquid media, inhibition of their growth by species-specific antisera and their sensitivity to digitonin (*Mycoplasma* spp. are digitonin sensitive and *Acholeplasma* spp. are not). Isolates were stored at -70°C.

Isolates were consequently identified as one of 7 types:

i) serologically identified *M.felis*

ii) serologically identified *M. equirhinis* and presumptive *M. equirhinis* based on growth characteristics, arginine metabolism and colony morphology

iii) *Acholeplasma* spp. based on insensitivity to digitonin

iv) slow growing, non-*M. felis* glucose fermenting *Mycoplasma* spp.

v) other arginine metabolising *Mycoplasma* spp.

vi) so-called 'D87-like' colonies based on morphology and biochemistry identical to a previous isolate which did not fit any classically described species

vii) other isolates.

## 3.5 Explanatory variables

The variables used in the analyses are described below in broad categories. The methods to derive microbiological and cytological variables have already been described in the description of the longitudinal methods. The variable names as used in the analyses and the values of their different levels are shown in parentheses.

### 3.5.1 Matching variables

Cases and 'all' controls were matched to one of the 7 trainers which contributed to the longitudinal study and to within 6 weeks of the date of sampling of the case. The trainer variable was categorical and all matched cases and controls were identified by a matching-set variable. Due to requirements for concurrent control selection and in order to maximise sample size, matching in this study was not 1:n but m:n (i.e. cases and control numbers varied between matched sets).

### 3.5.2 Time-associated, racing and sex variables

*3.5.2.1 Age*

The ages of horses at the time of examination were recorded as the age in years from January 1st of the year of birth up to January 1st of the year examination. All horses were between one (yearling) and eight years old, with the majority being 2 or 3 years old.

Age was classified as a categorical variable (AGECODE; 1-4) to broadly reflect the distribution of different age groups in these flat-training yards. The 4 categories were: (1) yearlings, (2) 2-year-olds, (3) 3-year-olds and (4) 4-year-olds and older.

Two-year-olds were treated as the referent group for the purposes of analyses since they had the largest numbers. Horses did not remain in the yearling category for long after entering training in the autumn of their yearling year and there was wastage of older horses (i.e. horses leaving study yards for any reason).

*3.5.2.2 Time since entering training*

The Racing Division of Weatherbys supplied dates of entry into training with the particular trainer for each horse on the study. Subtracting the date of entry into training from the date of sampling provided the period in days (ENTDD) that the horse had been in training with that trainer. Risk of disease and different periods of time in training were examined graphically to assess the most suitable way to express levels of this variable (Figure A1.1 in Appendix 1). There was evidence of a non-linear relationship between the logit of disease risk and period of time in training, as the risk of disease decreased initially with time and then levelled off. Therefore this continuous variable was transformed into a categorical variable (ENTDCO1; 0-2) to reflect time measured in months. The 3 categories were for horses that entered training less than one month ago (0), between one and 3 months ago (1) and more than 3 months ago (2).

*3.5.2.3 Previous racing*

The Racing Division of Weatherbys also supplied details of racing history for horses on the study where this was available. Horses were classified according to whether they had raced previously at the time of sampling (PREVRACE; 0-1). Where horses had raced previously, the date of their last race was subtracted from the sampling date to generate the

number of days since last raced (RACEDD). The relationship between risk of disease and time since last raced was again examined graphically to assess the best method of representing these data (Figure A1.2 in Appendix 1). Data examination showed that the logit of disease risk was not linear in relation to time since previous race and this variable was transformed into a categorical variable (RACDCOD; 0-2). Horses that had never raced before sampling were used as the referent group (0) with the other groups comprising horses that had raced within 7 days of sampling (1) and those that had raced more than a week previously (2). These data were represented in the same categories as those used by Christley *et al.* (1999; 2001a; 2001b).

*3.5.2.4 Sex*

The exact dates that colts were gelded were not recorded so the sex of horses were classified as male or female only (SEXCODE; 0-1), with females used as the referent group (0).

### 3.5.3 Microbiological variables

*3.5.3.1 Bacteria*

Tracheal wash bacteriology results were classified on the basis of total bacterial numbers isolated as well as numbers of individual species of bacteria. Total bacterial count, expressed as $\log_{10}$ colony forming units (cfu) per ml of tracheal wash was used as an ordered categorical variable with $\log_{10}$ cfu/ml bacteria rounded down to the nearest whole $\log_{10}$. Counts greater than 6 $\log_{10}$s were pooled in a single group because of low numbers of individuals in these categories.

Risk of disease and $\log_{10}$ cfu/ml numbers of individual bacterial species including mycoplasmas were examined graphically to assess the most suitable way to express variable levels for the purposes of logistic modelling (Figures A1.4 - A1.10 in Appendix 1).

There was a satisfactory linear relationship between the logit of disease risk and $log_{10}$ cfu/ml bacteria for *Streptococcus zooepidemicus* (SZOOC) and *Actinobacillus/Pasteurella* spp. (PASTALLC) and the risk of disease was expressed per $log_{10}$ cfu/ml increase relative to no bacterial culture for these variables. The relationship between logit disease risk and non-haemolytic *Streptococcus* spp. (NHSGP3; 0-2), *Staphylococcus* spp. (STAPHGP; 0-2) and *Acinetobacter* spp. (ACINETOG; 0-2) was best reflected by ordered categories (Table 4.2). Due to too few individuals within different categories, the remaining tracheal wash bacterial isolates were classified as binary variables, irrespective of bacterial numbers. No bacterial culture (0) was used as the referent group against culture of the organism (1) from the tracheal wash sample.

*3.5.3.2 Mycoplasma*

Three species of Mycoplasma, *Mycoplasma felis* (MFELIS), non-felis glucose fermenting *Mycoplasma* spp. (MNFGF) and *Mycoplasma equirhinis* (MEQUIR), were also classified as binary variables, cultured (1) or not cultured (0), because there were few individuals with different $log_{10}$ cfu/ml numbers of these bacteria.

*3.5.3.3 Nasopharyngeal swabs*

Results of nasopharyngeal swab cultures were classified as binary variables according to presence (1) or absence (0) of bacterial species cultured from swabs.

*3.5.3.4 Serology*

Seroconversion between paired blood samples taken at the time of initial examination and then several weeks later, was taken to indicate the presence of viral infection around the time of first sampling. Viral infection variables (equine herpesvirus (EHV), equine rhinovirus infection (ERV), equine adenovirus (ADENO) and equine

influenza (INFLUENZ)) were expressed as binary variables with seroconversion (1) compared with no seroconversion (0).

### 3.5.4 Endoscopy and cytology variables

#### *3.5.4.1 Tracheal mucopus*

Endoscopically visible tracheal mucopus was classified as an ordered categorical variable (MP; 0-3) with no mucopus visible (0), slight (1), moderate (2) or severe visible mucopus (3) (Figure 3.1; 3.1.1.3 Routine monthly examination and sampling).

#### *3.5.4.2 Tracheal haemorrhage*

Tracheal haemorrhage was classified as a binary variable (HAEMTRGP; 0-1) with presence (1) or absence of any visible haemorrhage (0) noted.

#### *3.5.4.3 Inflammation score*

Inflammation score as a measure of airway inflammation aggregated from endoscopic and cytological parameters has been described earlier (3.3.2.1 Inflammation score and 3.4.1 Cytology).

## 3.6 Data management

Following collation from the various laboratory and other sources, data from cases of clinically apparent respiratory disease were entered into a computer database using Epi Info 6 version 6.04b software (Dean *et al.*, 1994). The case database was designed to only allow entry of meaningful data by use of legal field limits through use of Epi Info CHECK files. Following entry, completed case data were checked for inconsistencies using appropriate frequency tabulations for variable values. Controls were randomly selected (subject to restrictions required for matching) from an existing longitudinal study dataset (Wood, 1999), which had already been validated and checked for inconsistencies. The

dataset for the case control study was then created by merging the datasets of clinically apparent cases and all selected controls, irrespective of endoscopic findings or cytological interpretation of tracheal washes. Merging of datasets was achieved using the CONCATENATE function from the MERGE menu in Epi Info.

Data were exported to Stata™ 5.0 software (Stata Corporation). Continuous and ordered categorical variables were transformed as appropriate into ordered categorical or binary variables. 'Healthy' and 'subclinical' controls were identified according to their inflammation score and 3 additional case variables (CASE2, CASE3 and CASE4) were created with horses being appropriately assigned to either of 2 levels for each variable, according to the status of horses being compared.

## 3.7 Statistical methods

### 3.7.1 Overview

Conditional logistic regression (CLR) is the appropriate regression technique for analysis of matched case control studies. In this type of analysis the probability of being a case is calculated in each stratum or matched set relative to the controls to which the case(s) is (are) matched i.e. a conditional probability or likelihood of being a case is calculated. The summary coefficient or relative risk measure for all cases is then calculated as the sum of probabilities over all strata that are concordant for exposure (i.e. strata that have all individuals classified the same way for exposure do not contribute to results). The conditional likelihood, $L_c$, where there are a variable number of controls per case in different strata may be represented as follows (Breslow & Day, 1980):

$$Lc = \prod_{i=1}^{I} \frac{1}{1 + \sum_{j=1}^{M_i} \exp\{\sum_{k=1}^{K} \beta_k (x_{ijk} - x_{i0k})\}}$$

Here the $i^{th}$ of I matched sets contains $M_i$ controls in addition to the cases and $x_{i0k}$ denotes the K-vector of exposures for the cases in this set and $x_{ijk}$ denotes the $k^{th}$ exposure for the $j^{th}$ control ($j$=1, 2, ...., $M_i$). It is clear from this conditional model that there is no estimate of an intercept term and there is no contribution to the likelihood estimate when all components of strata are concordant with respect to outcome status.

Classification of controls into 2 subgroups according to their tracheal inflammation scores meant that factors associated with different case and control definitions (datasets) could be determined. Comparison of the factors associated with each dataset would establish whether different factors were likely to be associated with clinically apparent and subclinical presentation of respiratory disease in this population of racehorses. The different datasets are briefly described below with the statistical approaches used. All statistical analyses were conducted using Stata™ 5.0 software (Stata Corporation).

### 3.7.2 Case control datasets

#### *3.7.2.1 Cases* vs *'all' controls*

'All' controls ('healthy' and 'subclinical' taken together) were compared with cases in the largest dataset. This approach would have the advantage of using a maximum sample size thus maximising the power of the study but controls, none of which had clinically apparent disease, were not differentiated according to tracheal inflammation score. This would mean that factors associated with a spectrum of clinical and subclinical disease would have the size of their effect reduced and possibly to the extent that that they would no longer be statistically significantly associated with clinically apparent disease.

#### *3.7.2.2 Cases* vs *'healthy' controls*

Use of a 'healthy' control group that included only horses without signs of airway inflammation, as measured by the aggregated inflammation score, would allow removal of

the effects of factors associated with subclinical respiratory disease. More clearly defined comparison groups would allow more reliable biological interpretation of findings but the exclusion of data, however, would reduce power and increase the size of an effect needed to be found statistically significant.

#### *3.7.2.3 Cases* vs *'subclinical' controls*

Use of a 'subclinical' control group that included only horses with signs of airway inflammation, as measured by the aggregated inflammation score, would allow direct evaluation of what factors were associated with clinical disease over and above those associated with the subclinical form. Again, more clearly defined comparison groups would maximise biological interpretation but with the cost of reducing power.

#### *3.7.2.4 'Healthy' controls* vs *'subclinical' controls*

'Subclinical' controls were here coded as cases and 'healthy' controls were used as the control group. This would determine which factors were associated with the presence of subclinical airway inflammation among the non-clinically affected horses and in turn allow better interpretation of earlier comparisons involving clinically apparent cases and both subgroups of controls.

### 3.7.3 Univariable analyses

All explanatory variables were examined for their univariable (crude) association with the probability of being a clinically apparent case by inclusion in a simple conditional logistic regression (CLR) model in which cases and controls were matched by set (trainer and time period). The association between outcome and each explanatory variable was expressed as a logistic regression coefficient ($\beta$) and standard error (S.E. $\beta$), an estimated crude odds ratio (OR) with 95% confidence intervals around the estimate (derived from

S.E. β), and corresponding Wald $\chi^2$ P-value. When the results of these analyses were cross-checked with those of conventional Mantel Haenszel methods used to adjust for the effects of matching, they were found to be very similar. Consequently only the results of the CLR models are presented.

### 3.7.4 Multivariable analyses

#### *3.7.4.1 Regression modelling strategy*

Following univariate analyses, 4 separate models were developed using multivariable conditional logistic regression modelling with the 4 different case and control definitions. This was undertaken to provide valid estimates of the strength of associations between significant explanatory variables and disease, having adjusted for the effects of other variables.

Variables believed *a priori* to be associated with disease and others that were associated with outcome in univariate analysis at a significance level $P<0.25$ were entered in conditional regression models during the model building process. An inclusion criteria of $P<0.25$ was chosen to allow variables that may be truly associated with diseases but were confounded by others at the univariate level, to be included in final models. Cytological markers of inflammation, including tracheal mucopus, inflammatory cell predominance, nucleated cell count and aggregated inflammation score and tracheal haemorrhage, were considered likely to be part of a causal pathway between infections and disease and were, therefore, not included as their inclusion in models with microbiological variables was likely to modify any effect towards unity. The matching process controlled factors confounding the association between clinically apparent disease and tracheal isolate variables. Due to the similarity of most housing and management of horses within training yards, most but not all factors related to housing, horse management and training were controlled by matching on

trainer and factors related to season and disease or infection clustering, were controlled by matching on time period.

A forward stepwise approach was adopted for multivariable analyses with systematic addition of *a priori* and significant main effects variables in descending order of their strength of association with the outcome. Variables were only retained if they were associated with disease (Wald $\chi^2$: $P \leq 0.05$) or if their inclusion resulted in a significant reduction in model deviance as measured by the likelihood ratio statistic (LRS $\chi^2$: $P \leq 0.05$) (Hosmer & Lemeshow, 1989). To ensure that statistically significant associations had not been inadvertently rejected, the final parsimonious model was checked by systematic addition of rejected variables one at a time.

Infection with influenza virus was extremely rare in this well vaccinated population and was not detected among 'healthy' controls. This presented particular problems with its inclusion in multivariable analyses involving this control group. In order to maximise available sample size and minimise the confounding effects from rare influenza infection, all analyses were conducted after exclusion of the 2 matched sets in which influenza seroconversions occurred.

Statistical interaction was examined by the addition of biologically meaningful, 2-way interaction terms between main effect variables. Interaction terms that provided a significant improvement to model fit as measured by likelihood ratio statistic (LRS $\chi^2$: $P \leq 0.05$) were retained.

*3.7.4.2 Post fit diagnostic procedures*

Final multivariable models were examined for goodness-of-fit using the Hosmer-Lemeshow technique (Hosmer & Lemeshow, 1989). The estimated probabilities from the models for each observation and the corresponding observed outcomes (taking the value one if a case and zero if a control) were ranked in ascending order of their estimated value

and split into ten groups corresponding to deciles of ascending risk. The sum of observed and predicted values for observations in each decile group were then calculated and the 2 × 10 contingency table tested for statistically significant differences across deciles using the modified chi-squared test with 8 degrees of freedom as described by Hosmer & Lemeshow (1989). A statistically significant result with $P<0.05$ in this test would indicate that there was poor prediction of outcome by the regression model.

In addition, final multivariable models were evaluated for their robustness by refitting of models following sequential exclusion of the observations with the largest delta-beta values (i.e. those observations whose exclusion were predicted to have the largest influence on each coefficient) for each variable included in the final model (Pregibon, 1981). Models were considered stable and robust when there was no significant effect on the fit of the model with exclusion of these observations as judged by the value and significance of coefficient estimates.

Finally, individual observations with the largest residual values, i.e. those observations whose outcomes were not well predicted by final models, were examined with respect to the variables included in these models. Contingency tables of case control status versus categories of included variables were generated for observations with absolute residual values >0.5 and the relative distribution of observations among these categories was examined.

# CHAPTER 4

# RESULTS

## 4.1 Description of data

Numbers and prevalence proportions of different categories of variables in clinically apparent cases, 'all' controls and subgroups of 'healthy' and 'subclinical' controls are presented in Tables 4.1 – 4.3.

Table 4.1 summarises the trainer details, age group, sex, endoscopic findings, inflammation score categories, time since entering yards and previous racing status for clinically apparent cases and 'all' controls, 'healthy' controls and 'subclinical' controls. Compared with 'all' controls a higher proportion of cases had positive grades of visible tracheal mucopus, had inflammation scores of 2 or 3 out of 3, were yearlings or 2-years-olds, female, had been in the yards less than 3 months and had not raced previously. Although numbers were small, a larger proportion of cases had visible tracheal haemorrhage compared to 'all' controls. Due to the requirements for matching, the relative proportions of cases and 'all' controls contributed by each trainer were very similar. However, the variation in relative proportions of subgroups of 'healthy' and 'subclinical' controls comprising 'all' controls contributed by each trainer reflected the overall prevalence of subclinical disease in each yard. As such, this would contribute a yard effect that was controlled for in matching by trainer. Predictably, the overall number of cases presented by each yard was correlated with the period that the yard was studied and the total number of horses in the yard. There were lower numbers of cases from the 2 yards in Lambourn and Epsom, due to the additional logistics involved in collecting samples from these yards because of their distance from the laboratories at the AHT. There was a slight seasonal variation in the presentation of cases, with fewer being seen between August and October than in the rest of the year when cases were more evenly presented. There was an increased

proportion of 'subclinical' controls seen between February and April compared to other periods.

Table 4.2 summarises the isolation of bacterial species from tracheal washes and their numbers. Results show that the proportion of horses with $\geq 10^3$ cfu/ml of total bacteria, *Pasteurella/Actinobacillus* spp., *S. zooepidemicus* and non-haemolytic *Streptococcus* spp. was larger amongst cases compared to 'all' controls and amongst 'subclinical' controls compared to 'healthy' controls. *S. pneumoniae* was isolated from a larger proportion of cases compared to 'all' controls and 'subclinical' controls compared to 'healthy' controls. In contrast, $<10^2$ cfu/ml (coagulase negative) *Staphylococcus* spp. were isolated from a larger proportion of 'all' controls compared to cases but in similar proportions from 'healthy' and 'subclinical' controls. Relatively few horses amongst both cases and controls had other species of bacteria isolated from tracheal washes.

Table 4.3 shows results of mycoplasma culture of tracheal washes available from 129 cases and 564 controls. There were relatively few horses from which mycoplasma were isolated from tracheal washes, although the proportion of horses from which *Mycoplasma felis* was isolated was higher amongst cases compared to 'all' controls. *Mycoplasma equirhinis* was the most frequently isolated *Mycoplasma* spp., from approximately equal proportions of cases and 'all' controls. Amongst controls, however, *M. equirhinis* was isolated more frequently from 'subclinical' controls.

Table 4.3 also includes nasopharyngeal swab bacterial culture and serology results for clinically apparent cases and the 3 control groups. Results of bacterial culture of nasopharyngeal swabs were available from 124 cases and 610 controls. The proportions of horses with bacteria isolated amongst all groups were broadly similar, although *S. zooepidemicus* was isolated from a marginally higher proportion of cases compared to 'all' controls and from more 'subclinical' controls than 'healthy' controls. Non-haemolytic

*Streptococcus* spp. and *Staphylococcus* spp. were isolated from more than 80% of all swabs from all groups.

Complement fixation (CF) tests were performed on samples from 141 cases and all 632 controls for equine herpes- and rhino viruses. Haemagglutination inhibition (HI) tests for equine adenovirus and equine influenza were performed on samples from 126 cases and all 632 controls. There were relatively few positive diagnoses of viral infection made, with less than 6 percent of horses in any group seroconverting to any single virus. Equine herpesvirus was the most common serologically diagnosed viral infection but was found in approximately equal proportions of acute cases and 'all' controls. Equine rhinovirus was diagnosed more frequently among cases than controls. Influenza was an extremely rare infection in this vaccinated population and was not diagnosed in 'healthy' controls throughout the duration of the study.

It may be seen that there were more missing data from cases than controls, especially for samples submitted for laboratory testing such as bacterial and mycoplasma culture and serology. This arose because clinical cases but not controls included horses that were sampled at times other than routine monthly yard visits usually by the yard's own veterinary surgeon. Consequently a larger proportion of these animals did not have a full set of samples submitted, despite attempts by study staff to encourage as complete sampling as possible.

## Table 4.1: Numbers and percentages of cases and controls for non-infectious explanatory variables

| *Explanatory variable* | | | | *Cases* | | *All Controls* | | *'Healthy' Controls* | | *'Subclinical' Controls* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *n* | *(%)* | *n* | *(%)* | *n* | *(%)* | *n* | *(%)* |
| ***Total numbers*** | | | | 170 | (100) | 632 | (100) | 304 | (100) | 328 | (100) |
| ***Trainer*** | ***Period studied*** | ***Total No. Horses*** | ***Location*** | | | | | | | | |
| 1 | 14 mths | 55-65 | Lambourn | 8 | (5) | 31 | (5) | 17 | (6) | 14 | (4) |
| 2 | 34 mths | 100-125 | Newmarket | 49 | (29) | 177 | (28) | 95 | (31) | 82 | (25) |
| 3 | 36 mths | 65 | Newmarket | 39 | (23) | 127 | (20) | 49 | (16) | 78 | (24) |
| 4 | 36 mths | 55-70 | Epsom | 13 | (8) | 52 | (8) | 35 | (12) | 17 | (5) |
| 5 | 27 mths | 70 | Newmarket | 32 | (19) | 125 | (20) | 68 | (22) | 57 | (17) |
| 6 | 12 mths | 175 | Newmarket | 15 | (9) | 60 | (9) | 15 | (5) | 45 | (14) |
| 7 | 6 mths | 90 | Newmarket | 14 | (8) | 60 | (9) | 25 | (8) | 35 | (11) |
| ***Season*** | | | | | | | | | | | |
| November – January | | | Winter | 47 | (27) | 169 | (27) | 84 | (28) | 85 | (26) |
| February – April | | | Spring | 46 | (27) | 196 | (31) | 80 | (26) | 116 | (35) |
| May – July | | | Summer | 47 | (28) | 162 | (26) | 85 | (28) | 77 | (23) |
| August – October | | | Autumn | 30 | (18) | 105 | (17) | 55 | (18) | 50 | (15) |
| ***Age group**** | | | Yearlings | 15 | (9) | 12 | (2) | 6 | (2) | 6 | (2) |
| | | | 2 year-olds | 98 | (58) | 326 | (52) | 151 | (50) | 175 | (53) |
| | | | 3 year-olds | 39 | (23) | 226 | (36) | 114 | (38) | 112 | (34) |
| | | | ≥4 year-olds | 18 | (11) | 68 | (11) | 33 | (11) | 35 | (11) |
| ***Sex*** | | | Female | 68 | (40) | 177 | (28) | 78 | (26) | 99 | (30) |
| | | | Male | 102 | (60) | 455 | (72) | 226 | (74) | 229 | (70) |
| ***Endoscopic examination**** | | | | | | | | | | | |
| Visible tracheal mucopus | | | none visible | 45 | (29) | 414 | (66) | 238 | (78) | 176 | (54) |
| | | | slight | 55 | (36) | 149 | (24) | 66 | (22) | 83 | (25) |
| | | | moderate | 43 | (28) | 61 | (10) | 0 | (0) | 61 | (19) |
| | | | severe | 10 | (7) | 8 | (1) | 0 | (0) | 8 | (2) |
| Visible tracheal haemorrhage | | | none visible | 139 | (92) | 620 | (98) | 298 | (98) | 322 | (98) |
| | | | visible | 12 | (8) | 12 | (2) | 6 | (2) | 6 | (2) |
| ***Inflammation score**** (out of 3) | | | 0 | 44 | (27) | 304 | (48) | 304 | (100) | 0 | (0) |
| | | | 1 | 55 | (34) | 238 | (38) | 0 | (0) | 238 | (73) |
| | | | 2 | 37 | (23) | 52 | (8) | 0 | (0) | 52 | (16) |
| | | | 3 | 28 | (17) | 38 | (6) | 0 | (0) | 38 | (12) |
| ***Time since entering training yard**** | | | <1 month | 21 | (13) | 27 | (4) | 10 | (3) | 17 | (5) |
| | | | 1-3 months | 34 | (21) | 76 | (12) | 30 | (10) | 46 | (14) |
| | | | >3 months | 110 | (67) | 517 | (83) | 259 | (87) | 258 | (80) |
| ***Raced previously*** | | | No | 107 | (63) | 323 | (51) | 136 | (45) | 187 | (57) |
| | | | Yes | 63 | (37) | 309 | (49) | 168 | (55) | 141 | (43) |
| ***Time since last race*** | | | Never raced | 107 | (63) | 323 | (51) | 136 | (45) | 187 | (57) |
| | | | 1-7 days | 8 | (5) | 52 | (8) | 26 | (9) | 26 | (8) |
| | | | >7 days | 55 | (32) | 257 | (41) | 142 | (47) | 115 | (35) |

*missing data values explain departures from totals

## Table 4.2: Numbers and percentages of cases and controls for tracheal wash (TW) bacterial culture explanatory variables

| *Explanatory variable* | | *Cases* | | *All Controls* | | *'Healthy' Controls* | | *'Subclinical' Controls* | |
|---|---|---|---|---|---|---|---|---|---|
| | | *n* | *(%)* | *n* | *(%)* | *n* | *(%)* | *n* | *(%)* |
| ***TW bacterial culture*** | TW submitted | 157 | (92) | 632 | (100) | 304 | (100) | 328 | (100) |
| | no TW submitted | 13 | (8) | 0 | (0) | 0 | (0) | 0 | (0) |
| Total bacterial count | none isolated | 22 | (14) | 149 | (24) | 90 | (30) | 59 | (18) |
| | $<10^2$ cfu/ml | 15 | (10) | 122 | (19) | 59 | (19) | 63 | (19) |
| | $10^2$-$10^3$ cfu/ml | 23 | (15) | 140 | (22) | 72 | (24) | 68 | (21) |
| | $10^3$-$10^4$ cfu/ml | 31 | (20) | 107 | (17) | 49 | (16) | 58 | (18) |
| | $10^4$-$10^5$ cfu/ml | 28 | (18) | 70 | (11) | 26 | (9) | 44 | (13) |
| | $10^5$-$10^6$ cfu/ml | 21 | (13) | 29 | (5) | 8 | (3) | 21 | (6) |
| | $>10^6$ cfu/ml | 17 | (11) | 15 | (2) | 0 | (0) | 15 | (5) |
| *Actinobacillus/Pasteurella spp.* | none isolated | 95 | (61) | 477 | (75) | 251 | (83) | 226 | (69) |
| | $<10^2$ cfu/ml | 3 | (2) | 36 | (6) | 15 | (5) | 21 | (60 |
| | $10^2$-$10^3$ cfu/ml | 16 | (10) | 41 | (6) | 16 | (5) | 25 | (8) |
| | $10^3$-$10^4$ cfu/ml | 11 | (7) | 37 | (6) | 12 | (4) | 25 | (8) |
| | $10^4$-$10^5$ cfu/ml | 9 | (6) | 28 | (4) | 8 | (3) | 20 | (6) |
| | $10^5$-$10^6$ cfu/ml | 13 | (8) | 8 | (1) | 2 | (<1) | 6 | (2) |
| | $>10^6$ cfu/ml | 10 | (6) | 5 | (<1) | 0 | (0) | 5 | (2) |
| *Streptococcus zooepidemicus* | none isolated | 85 | (54) | 446 | (71) | 240 | (79) | 206 | (63) |
| | $<10^2$ cfu/ml | 18 | (11) | 77 | (12) | 31 | (10) | 46 | (14) |
| | $10^2$-$10^3$ cfu/ml | 15 | (10) | 53 | (8) | 21 | (7) | 32 | (10) |
| | $10^3$-$10^4$ cfu/ml | 11 | (7) | 28 | (4) | 9 | (3) | 19 | (6) |
| | $10^4$-$10^5$ cfu/ml | 9 | (6) | 18 | (3) | 3 | (1) | 15 | (5) |
| | $10^5$-$10^6$ cfu/ml | 11 | (7) | 7 | (1) | 0 | (0) | 7 | (2) |
| | $>10^6$ cfu/ml | 8 | (5) | 3 | (<1) | 0 | (0) | 3 | (1) |
| *Streptococcus pneumoniae* | not isolated | 135 | (86) | 580 | (92) | 289 | (95) | 291 | (89) |
| | isolated | 22 | (14) | 52 | (8) | 15 | (5) | 37 | (11) |
| *Streptococcus equisimilis* | not isolated | 150 | (96) | 621 | (98) | 298 | (98) | 323 | (98) |
| | isolated | 7 | (4) | 11 | (2) | 6 | (2) | 5 | (2) |
| Non-haem. *Streptococcus* spp. | $<10^3$ cfu/ml | 103 | (66) | 490 | (78) | 250 | (82) | 240 | (73) |
| | $10^3$-$10^4$ cfu/ml | 37 | (24) | 123 | (19) | 50 | (16) | 73 | (22) |
| | $>10^4$ cfu/ml | 17 | (11) | 19 | (3) | 4 | (1) | 15 | (5) |
| *Acinetobacter* spp. | not isolated | 149 | (95) | 581 | (92) | 288 | (95) | 293 | (89) |
| | $<10^2$ cfu/ml | 4 | (3) | 37 | (6) | 12 | (4) | 25 | (8) |
| | $>10^2$ cfu/ml | 4 | (3) | 14 | (2) | 4 | (1) | 10 | (3) |
| *Bacillus* spp. | not isolated | 147 | (94) | 597 | (94) | 290 | (95) | 307 | (94) |
| | isolated | 10 | (6) | 35 | (6) | 14 | (5) | 21 | (6) |
| *Bordetella bronchiseptica* | not isolated | 156 | (>99) | 627 | (>99) | 301 | (>99) | 326 | (>99) |
| | isolated | 1 | (<1) | 5 | (<1) | 3 | (<1) | 2 | (<1) |
| *Enterobacter* spp. | not isolated | 155 | (99) | 624 | (99) | 299 | (98) | 325 | (99) |
| | isolated | 2 | (1) | 8 | (1) | 50 | (2) | 3 | (1) |
| *Escherichia coli* | not isolated | 148 | (94) | 603 | (95) | 293 | (96) | 310 | (95) |
| | isolated | 9 | (6) | 29 | (5) | 11 | (4) | 18 | (5) |
| *Pseudomonas* spp. | not isolated | 156 | (>99) | 631 | (>99) | 303 | (>99) | 328 | (100) |
| | isolated | 1 | (<1) | 1 | (<1) | 1 | (<1) | 0 | (0) |
| *Serratia* spp. | not isolated | 156 | (>99) | 627 | (>99) | 303 | (>99) | 324 | (99) |
| | isolated | 1 | (<1) | 5 | (<1) | 1 | (<1) | 4 | (1) |
| *Staphylococcus* spp. | not isolated | 113 | (72) | 371 | (59) | 183 | (60) | 188 | (57) |
| | $<10^2$ cfu/ml | 15 | (10) | 136 | (22) | 70 | (23) | 66 | (20) |
| | $>10^2$ cfu/ml | 29 | (18) | 125 | (20) | 51 | (17) | 74 | (23) |

## Table 4.3: Numbers and percentages of cases and controls for tracheal wash (TW) mycoplasma and nasopharyngeal swab bacterial culture and serological explanatory variables

| *Explanatory variable* | | *Cases* | | *All Controls* | | *'Healthy' Controls* | | *'Subclinical' Controls* | |
|---|---|---|---|---|---|---|---|---|---|
| | | *n* | *(%)* | *n* | *(%)* | *n* | *(%)* | *n* | *(%)* |
| ***TW Mycoplasma culture*** | TW submitted | 129 | (76) | 564 | (89) | 271 | (89) | 293 | (89) |
| | no TW submitted | 41 | (24) | 68 | (11) | 33 | (11) | 35 | (11) |
| *Mycoplasma felis* | not isolated | 119 | (92) | 557 | (99) | 270 | (>99) | 287 | (98) |
| | isolated | 10 | (8) | 7 | (1) | 1 | (<1) | 6 | (2) |
| NFGF *Mycoplasma* spp. | not isolated | 122 | (95) | 555 | (98) | 266 | (98) | 289 | (99) |
| | isolated | 7 | (5) | 9 | (2) | 5 | (2) | 4 | (1) |
| *Mycoplasma equirhinis* | not isolated | 111 | (86) | 494 | (88) | 250 | (92) | 246 | (84) |
| | isolated | 18 | (14) | 68 | (12) | 21 | (8) | 47 | (16) |
| ***NP swab bacterial culture*** | swab submitted | 124 | (73) | 610 | (97) | 298 | (98) | 312 | (95) |
| | no swab submitted | 46 | (27) | 22 | (3) | 6 | (2) | 16 | (5) |
| *Pasteurella* spp. | not isolated | 80 | (65) | 428 | (70) | 213 | (71) | 215 | (69) |
| | isolated | 44 | (35) | 182 | (30) | 85 | (29) | 97 | (31) |
| *S. zooepidemicus* | not isolated | 76 | (61) | 438 | (72) | 224 | (75) | 214 | (69) |
| | isolated | 48 | (39) | 172 | (28) | 74 | (25) | 98 | (31) |
| *S. pneumoniae* | not isolated | 109 | (88) | 537 | (88) | 269 | (90) | 268 | (86) |
| | isolated | 15 | (12) | 73 | (12) | 29 | (10) | 44 | (14) |
| Non-haem. *Strep.* spp. | not isolated | 22 | (18) | 71 | (12) | 30 | (10) | 41 | (13) |
| | isolated | 102 | (82) | 539 | (88) | 268 | (90) | 271 | (87) |
| *Acinetobacter* spp. | not isolated | 99 | (80) | 460 | (75) | 231 | (78) | 229 | (73) |
| | isolated | 25 | (20) | 150 | (25) | 67 | (22) | 83 | (27) |
| *Bacillus* spp. | not isolated | 109 | (87) | 523 | (86) | 255 | (86) | 268 | (86) |
| | isolated | 16 | (13) | 87 | (14) | 43 | (14) | 44 | (14) |
| *Enterobacter* spp. | not isolated | 122 | (98) | 598 | (98) | 292 | (98) | 306 | (98) |
| | isolated | 2 | (2) | 12 | (2) | 6 | (2) | 6 | (2) |
| *Escherichia coli* | not isolated | 120 | (97) | 597 | (98) | 291 | (98) | 306 | (98) |
| | isolated | 4 | (3) | 13 | (2) | 7 | (2) | 6 | (2) |
| *Serratia* spp. | not isolated | 123 | (>99) | 603 | (99) | 293 | (98) | 310 | (>99) |
| | isolated | 1 | (<1) | 7 | (1) | 5 | (2) | 2 | (<1) |
| *Staphylococcus* spp. | not isolated | 20 | (16) | 93 | (15) | 45 | (15) | 48 | (15) |
| | isolated | 104 | (84) | 517 | (85) | 253 | (85) | 264 | (85) |
| ***Serology*** | pairs CF tested | 141 | (83) | 632 | (100) | 304 | (100) | 328 | (100) |
| | pairs HI tested | 126 | (74) | 632 | (100) | 304 | (100) | 328 | (100) |
| EHV-1 & EHV-4 (CF) | no seroconversion | 133 | (94) | 609 | (96) | 294 | (97) | 315 | (96) |
| | seroconversion | 8 | (6) | 23 | (4) | 10 | (3) | 13 | (4) |
| ERV-1 (CF) | no seroconversion | 137 | (96) | 623 | (99) | 301 | (99) | 322 | (98) |
| | seroconversion | 4 | (4) | 9 | (1) | 3 | (1) | 6 | (2) |
| Equine adenovirus (HI) | no seroconversion | 125 | (>99) | 627 | (>99) | 302 | (>99) | 325 | (>99) |
| | seroconversion | 1 | (<1) | 5 | (<1) | 2 | (<1) | 3 | (<1) |
| Equine influenza virus (HI) | no seroconversion | 123 | (98) | 630 | (>99) | 304 | (100) | 326 | (>99) |
| | seroconversion | 3 | (2) | 2 | (<1) | 0 | (0) | 2 | (<1) |

NFGF = Non-felis glucose fermenting

## 4.2 Cases *vs* 'all' controls

### 4.2.1 Univariable analyses

Table 4.4 summarises univariable associations between clinically apparent respiratory disease and non-infectious explanatory variables using 'all' controls. Horses were at significantly increased risk of being a case with younger age, being female, increasing mucopus and inflammation scores and presence of tracheal haemorrhage. Risk was increased in horses that had never raced previously but decreased with the time they had been in the training yard.

**Table 4.4: Univariable associations between clinically apparent cases and non-infectious explanatory variables using 'all' controls**

| *Explanatory variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Age group*** | Yearlings | 11.6 | 3.5 – 38.0 | <0.001 |
| | 2 year-olds | referent | | |
| | 3 year-olds | 0.5 | 0.3 – 0.8 | 0.002 |
| | ≥4 year-olds | 0.8 | 0.4 – 1.5 | 0.458 |
| ***Sex*** | Female | referent | | |
| | Male | 0.6 | 0.4 – 0.8 | 0.002 |
| ***Visible tracheal mucopus*** | none visible | referent | | |
| | slight | 4.3 | 2.7 – 6.9 | <0.001 |
| | moderate | 9.9 | 5.6 – 17.6 | <0.001 |
| | severe | 24.3 | 8.2 – 71.8 | <0.001 |
| ***Visible tracheal haemorrhage*** | none visible | referent | | |
| | visible | 4.7 | 2.0 – 11.1 | <0.001 |
| ***Inflammation score*** (out of 3) | 0 | referent | | |
| | 1 | 1.6 | 1.0 – 2.5 | 0.037 |
| | 2 | 6.6 | 3.7 – 11.7 | <0.001 |
| | 3 | 7.2 | 3.8 – 13.7 | <0.001 |
| ***Time since entering training yard*** | <1 month | 4.5 | 2.3 – 9.1 | <0.001 |
| | 1-3 months | 3.3 | 1.8 – 5.8 | <0.001 |
| | >3 months | referent | | |
| ***Raced previously*** | No | referent | | |
| | Yes | 0.6 | 0.4 – 0.8 | 0.004 |
| ***Time since last race*** | Never raced | referent | | |
| | 1-7 days | 0.4 | 0.2 – 1.0 | 0.040 |
| | >7 days | 0.6 | 0.4 – 0.9 | 0.010 |

Table 4.5 shows that clinically apparent respiratory disease was associated with several microbiological variables in univariable analyses. The risk of being a case increased with greater $\log_{10}$ cfu/ml of tracheal wash for total bacterial count, *Actinobacillus/Pasteurella* spp. and *S. zooepidemicus.* Non-haemolytic *Streptococcus* spp. at $>10^4$ cfu/ml of tracheal wash was significantly associated with clinical disease as was the presence of *S. pneumoniae, M. felis* and non-felis glucose fermenting (NFGF) *Mycoplasma* spp.. *Staphylococcus* spp. at $<10^2$ cfu/ml of tracheal wash was inversely associated with the risk of clinically apparent respiratory disease. Infections with known equine viruses were not significantly associated with clinical disease other than equine influenza, which had the largest OR of 7.0 being marginally significant (P = 0.051). The presence of non-haemolytic *Streptococcus* spp in NP swabs was inversely associated with disease.

## Table 4.5: Univariable associations between clinically apparent cases and microbiological explanatory variables using 'all' controls

| *Explanatory variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Tracheal wash bacterial isolates*** | | | | |
| Total bacterial count | none isolated | referent | | |
| | $<10^2$ cfu/ml | 0.9 | 0.4 – 1.8 | 0.668 |
| | $10^2$-$10^3$ cfu/ml | 1.2 | 0.6 – 2.3 | 0.610 |
| | $10^3$-$10^4$ cfu/ml | 2.1 | 1.1 – 4.0 | 0.023 |
| | $10^4$-$10^5$ cfu/ml | 3.3 | 1.7 – 6.4 | 0.001 |
| | $10^5$-$10^6$ cfu/ml | 6.1 | 2.8 – 13.5 | <0.001 |
| | $>10^6$ cfu/ml | 9.2 | 3.8 – 22.6 | <0.001 |
| *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 1.4 | 1.3 – 1.6 | <0.001 |
| *Streptococcus zooepidemicus* | log cfu/ml$^{-1}$ | 1.4 | 1.3 – 1.6 | <0.001 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 1.8 | 1.0 – 3.2 | 0.034 |
| *Streptococcus equisimilis* | not isolated | referent | | |
| | isolated | 2.4 | 0.9 – 6.5 | 0.074 |
| Non-haemolytic *Streptococcus* spp. | $<10^3$ cfu/ml | referent | | |
| | $10^3$-$10^4$ cfu/ml | 1.4 | 0.9 – 2.2 | 0.141 |
| | $>10^4$ cfu/ml | 4.2 | 2.0 – 8.6 | <0.001 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 0.4 | 0.1 – 1.1 | 0.087 |
| | $>10^2$ cfu/ml | 1.0 | 0.3 – 3.4 | 0.937 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 1.2 | 0.6 – 2.5 | 0.651 |
| *Bordetella bronchiseptica* | not isolated | referent | | |
| | isolated | 0.6 | 0.06 – 6.2 | 0.680 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 0.9 | 0.2 – 4.7 | 0.886 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 1.3 | 0.6 – 2.8 | 0.563 |
| *Pseudomonas* spp. | not isolated | referent | | |
| | isolated | 3.6 | 0.2 – 57.4 | 0.368 |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 0.8 | 0.1 – 6.9 | 0.837 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 0.3 | 0.2 – 0.6 | <0.001 |
| | $>10^2$ cfu/ml | 0.7 | 0.5 – 1.2 | 0.181 |
| ***Tracheal wash Mycoplasma isolates*** | | | | |
| *Mycoplasma felis* | not isolated | referent | | |
| | isolated | 5.3 | 1.7 – 17.1 | 0.005 |
| NFGF *Mycoplasma* spp. | not isolated | referent | | |
| | isolated | 3.6 | 1.2 – 10.3 | 0.019 |
| *Mycoplasma equirhinis* | not isolated | referent | | |
| | isolated | 1.2 | 0.7 – 2.1 | 0.578 |

NFGF = Non-felis glucose fermenting

Table 4.5 continued

| *Variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Serological evidence of viral infection*** | | | | |
| Equine herpesvirus | no seroconversion | referent | | |
| | seroconversion | 1.5 | 0.5 – 4.0 | 0.440 |
| Equine rhinovirus | no seroconversion | referent | | |
| | seroconversion | 2.1 | 0.6 – 7.2 | 0.239 |
| Adenovirus | no seroconversion | referent | | |
| | seroconversion | 1.0 | 0.1 – 9.4 | 0.978 |
| Influenza | no seroconversion | referent | | |
| | seroconversion | 7.0 | 1.0 – 50.2 | 0.051 |
| ***Nasopharyngeal swab bacterial isolates*** | | | | |
| *Pasteurella* spp. | not isolated | referent | | |
| | isolated | 1.4 | 0.9 – 2.4 | 0.168 |
| *Streptococcus zooepidemicus* | not isolated | referent | | |
| | isolated | 1.5 | 1.0 – 2.3 | 0.068 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 0.9 | 0.5 – 1.6 | 0.633 |
| Non-haemolytic *Streptococcus* spp. | not isolated | referent | | |
| | isolated | 0.6 | 0.3 – 1.0 | 0.037 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | isolated | 0.73 | 0.4 – 1.3 | 0.258 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 0.9 | 0.5 – 1.6 | 0.600 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 0.8 | 0.2 – 3.9 | 0.789 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 2.0 | 0.6 – 6.8 | 0.286 |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 0.6 | 0.07 – 4.7 | 0.613 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | isolated | 1.0 | 0.6 – 1.7 | 0.949 |

### 4.2.2 Multivariable analyses

Table 4.6 summarises the final multivariable CLR model for clinically apparent respiratory disease using 'all' controls, excluding the 2 matched sets in which influenza infections occurred. After controlling for microbiological variables, clinical respiratory disease remained significantly associated with age and time since entry into the training yard; with younger horses and those that had been in training less than 3 months at increased risk. Risk of being a case was significantly and positively associated with presence of *Actinobacillus/Pasteurella* spp. and *M. felis* in tracheal washes but was inversely associated with *Staphylococcus* and *Acinetobacter* spp.. No statistically significant interactions were identified in this model.

**Table 4.6: Final multivariable CLR model for associations between clinically apparent respiratory disease and explanatory variables using 'all' controls**

| *Explanatory variable* | | β | S.E. β | *Adjusted OR* | *95% CI* | *P-value* |
|---|---|---|---|---|---|---|
| Age group | Yearlings | 2.55 | 1.17 | 12.8 | 1.3 – 128 | 0.029 |
| | 2 year-olds | referent | | 1.0 | | |
| | 3 year-olds | -0.51 | 0.31 | 0.6 | 0.3 – 1.1 | 0.098 |
| | ≥4 year-olds | -0.24 | 0.43 | 0.8 | 0.3 – 1.8 | 0.576 |
| | | | | | | **0.028* |
| Time since entering training yard | <1 month | 1.71 | 0.59 | 5.5 | 1.7 – 17.6 | 0.004 |
| | 1-3 months | 0.89 | 0.39 | 2.4 | 1.1 – 5.2 | 0.022 |
| | >3 months | referent | | 1.0 | | |
| | | | | | | **0.005* |
| TW *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 0.26 | 0.08 | 1.3 | 1.1 – 1.5 | 0.001 |
| | | | | | | **0.0006* |
| TW *Staphylococcus* spp. | not isolated | referent | | 1.0 | | |
| | <$10^2$ cfu/ml | -0.97 | 0.38 | 0.4 | 0.2 – 0.8 | 0.010 |
| | >$10^2$ cfu/ml | -0.01 | 0.28 | 1.0 | 0.6 – 1.7 | 0.981 |
| | | | | | | **0.020* |
| TW *Acinetobacter* spp. | not isolated | referent | | 1.0 | | |
| | <$10^2$ cfu/ml | -1.25 | 0.06 | 0.3 | 0.1 – 0.9 | 0.030 |
| | >$10^2$ cfu/ml | -0.04 | 0.69 | 1.0 | 0.3 – 3.6 | 0.956 |
| | | | | | | **0.048* |
| TW *M. felis* | not isolated | referent | | 1.0 | | |
| | isolated | 1.44 | 0.67 | 4.2 | 1.1 – 15.9 | 0.033 |
| | | | | | | **0.029* |

*Likelihood ratio statistic $\chi^2$ P-value TW = tracheal wash isolate
Hosmer-Lemeshow $\chi^2$ = 3.45; P = 0.90

## 4.3 Cases *vs* 'healthy' controls

### 4.3.1 Univariable analyses

Table 4.7 summarises univariable associations between clinically apparent respiratory disease and non-infectious explanatory variables using 304 'healthy' controls. Using this set of controls, horses were at increased risk of being a clinical case for the same factors as for 'all' controls (younger age, female, tracheal haemorrhage, less time in training yard and not raced previously). However, the strengths of the associations were generally larger than when 'all' controls were used.

**Table 4.7: Univariable associations between clinically apparent cases and non-infectious explanatory variables using 'healthy' controls**

| *Variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Age group*** | Yearlings | 13.4 | 3.3 – 54.3 | <0.001 |
| | 2 year-olds | referent | | |
| | 3 year-olds | 0.4 | 0.3 – 0.7 | 0.001 |
| | ≥4 year-olds | 0.9 | 0.4 – 1.8 | 0.702 |
| ***Sex*** | Female | referent | | |
| | Male | 0.5 | 0.3 – 0.8 | 0.002 |
| ***Visible tracheal haemorrhage*** | none visible | referent | | |
| | visible | 5.3 | 1.8 – 15.4 | 0.002 |
| ***Time since entering training yard*** | <1 month | 5.9 | 2.5 – 14.1 | <0.001 |
| | 1-3 months | 4.1 | 2.0 – 8.3 | <0.001 |
| | >3 months | referent | | |
| ***Raced previously*** | No | referent | | |
| | Yes | 0.5 | 0.3 – 0.7 | 0.001 |
| ***Time since last race*** | Never raced | referent | | |
| | 1-7 days | 0.5 | 0.2 – 1.1 | 0.084 |
| | >7 days | 0.5 | 0.3 – 0.7 | 0.001 |

Table 4.8 shows that with univariable analysis, clinically apparent cases of respiratory disease compared to 'healthy' controls were significantly associated with the same tracheal wash bacterial variables as 'all' controls, but the strength of the associations were increased. The association between *M. felis* and clinical disease was increased compared to 'all' controls although smaller sample numbers gave rise to very wide confidence intervals. NFGF *Mycoplasma* spp. and *M. equirhinis* were not associated with disease. *Staphylococcus* spp. in tracheal washes at $<10^2$ cfu/ml demonstrated an inverse association with disease. In this analysis the presence of *S. zooepidemicus* in nasopharyngeal swabs was associated with disease but non-haemolytic *Streptococcus* spp. in swabs was inversely associated with clinical disease. There was again no association with viral infections and no diagnoses of influenza were made amongst 'healthy' controls.

## Table 4.8: Univariable associations between clinically apparent cases and microbiological explanatory variables using 'healthy' controls

| *Explanatory variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Tracheal wash bacterial isolates*** | | | | |
| Total bacterial count | none isolated | referent | | |
| | $<10^2$ cfu/ml | 1.1 | 0.5 – 2.3 | 0.874 |
| | $10^2$-$10^3$ cfu/ml | 1.4 | 0.7 – 2.9 | 0.304 |
| | $10^3$-$10^4$ cfu/ml | 2.2 | 1.1 – 4.6 | 0.025 |
| | $10^4$-$10^5$ cfu/ml | 4.3 | 2.0 – 9.3 | <0.001 |
| | $10^5$-$10^6$ cfu/ml | 8.7 | 3.2 – 23.8 | <0.001 |
| | $>10^6$ cfu/ml | ∞ | – | – |
| *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 1.6 | 1.3 – 1.8 | <0.001 |
| *Streptococcus zooepidemicus* | log cfu/ml$^{-1}$ | 1.7 | 1.4 – 2.0 | <0.001 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 3.0 | 1.5 – 6.3 | 0.002 |
| *Streptococcus equisimilis* | not isolated | referent | | |
| | isolated | 2.3 | 0.7 – 7.3 | 0.149 |
| Non-haemolytic *Streptococcus* spp. | $<10^3$ cfu/ml | referent | | |
| | $10^3$-$10^4$ cfu/ml | 1.6 | 1.0 – 2.7 | 0.067 |
| | $>10^4$ cfu/ml | 9.4 | 2.9 – 30.5 | <0.001 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 0.6 | 0.2 – 1.9 | 0.373 |
| | $>10^2$ cfu/ml | 1.6 | 0.3 – 7.5 | 0.561 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 1.9 | 0.8 – 4.8 | 0.162 |
| *Bordetella bronchiseptica* | not isolated | referent | | |
| | isolated | 0.4 | 0.03 – 4.6 | 0.437 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 0.6 | 0.1 – 3.5 | 0.555 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 1.8 | 0.7 – 4.6 | 0.241 |
| *Pseudomonas* spp. | not isolated | referent | | |
| | isolated | 1.7 | 0.1 – 27.0 | 0.717 |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 1.2 | 0.1 – 18.9 | 0.92 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 0.3 | 0.2 – 0.6 | 0.001 |
| | $>10^2$ cfu/ml | 0.8 | 0.5 – 1.4 | 0.432 |
| ***Tracheal wash Mycoplasma isolates*** | | | | |
| *Mycoplasma felis* | not isolated | referent | | |
| | isolated | 11.6 | 1.3 – 102.4 | 0.028 |
| NFGF *Mycoplasma* spp. | not isolated | referent | | |
| | isolated | 3.0 | 0.9 – 10.7 | 0.086 |
| *Mycoplasma equirhinis* | not isolated | referent | | |
| | isolated | 1.9 | 0.9 – 4.0 | 0.101 |

NFGF = Non-felis glucose fermenting

Table 4.8 continued

| *Variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Serological evidence of viral infection*** | | | | |
| Equine herpesvirus | no seroconversion | referent | | |
| | seroconversion | 1.8 | 0.6 – 5.7 | 0.305 |
| Equine rhinovirus | no seroconversion | referent | | |
| | seroconversion | 4.0 | 0.8 – 20.3 | 0.090 |
| Adenovirus | no seroconversion | referent | | |
| | seroconversion | 0.8 | 0.1 – 9.5 | 0.894 |
| Influenza | no seroconversion | referent | | |
| | seroconversion | ∞ | – | – |
| ***Nasopharyngeal swab bacterial isolates*** | | | | |
| *Pasteurella* spp. | not isolated | referent | | |
| | isolated | 1.6 | 0.9 – 2.7 | 0.128 |
| *Streptococcus zooepidemicus* | not isolated | referent | | |
| | isolated | 1.8 | 1.1 – 2.9 | 0.019 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 1.1 | 0.5 – 2.2 | 0.890 |
| Non-haemolytic *Streptococcus* spp. | not isolated | referent | | |
| | isolated | 0.5 | 0.2 – 0.9 | 0.028 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | isolated | 0.8 | 0.4 – 1.5 | 0.522 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 1.0 | 0.5 – 1.9 | 0.928 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 0.9 | 0.2 – 5.0 | 0.929 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 2.2 | 0.5 – 10.1 | 0.326 |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 0.4 | 0.05 – 3.8 | 0.451 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | isolated | 0.9 | 0.5 – 1.8 | 0.862 |

### 4.3.2 Multivariable analyses

Table 4.9 summarises the final multivariable CLR model for clinically apparent respiratory disease using only 'healthy' controls, excluding the 2 matched sets in which influenza infections occurred. After controlling for microbiological variables, clinical respiratory disease remained significantly associated with age and time since entry into the training yard; with younger horses and those that had been in training less than 3 months at increased risk. Risk of being a case was significantly and positively associated with presence of *Actinobacillus/Pasteurella* spp. and *S. zooepidemicus* in tracheal washes, and *Actinobacillus/Pasteurella* spp. in nasopharyngeal swabs but was inversely associated with

*Staphylococcus* spp. in washes and non-haemolytic *Streptococcus* spp. in swabs. No statistically significant interactions were identified in this model.

**Table 4.9: Final multivariable CLR model for associations between clinically apparent respiratory disease and explanatory variables using 'healthy' controls**

| *Explanatory variable* | | β | S.E.β | *Adjusted OR* | *95% CI* | *P-value* |
|---|---|---|---|---|---|---|
| Age group | Yearlings | 2.64 | 1.30 | 14.0 | 1.1 – 179 | 0.042 |
| | 2 year-olds | referent | | 1.0 | | |
| | 3 year-olds | -0.74 | 0.39 | 0.5 | 0.2 – 1.0 | 0.057 |
| | ≥4 year-olds | 0.10 | 0.53 | 1.1 | 0.4 – 3.1 | 0.849 |
| | | | | | | **0.020* |
| Time since entering training yard | <1 month | 1.23 | 0.67 | 3.4 | 0.9 – 12.6 | 0.065 |
| | 1-3 months | 1.47 | 0.51 | 4.3 | 1.6 – 11.7 | 0.004 |
| | >3 months | referent | | 1.0 | | |
| | | | | | | **0.006* |
| TW *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 0.38 | 0.12 | 1.5 | 1.1 – 1.9 | 0.002 |
| | | | | | | **0.0017* |
| TW *S. zooepidemicus* | log cfu/ml$^{-1}$ | 0.28 | 0.13 | 1.3 | 1.0 – 1.7 | 0.029 |
| | | | | | | **0.024* |
| TW *Staphylococcus* spp. | not isolated | referent | | 1.0 | | |
| | <$10^2$ cfu/ml | -0.96 | 0.45 | 0.4 | 0.2 – 0.9 | 0.032 |
| | >$10^2$ cfu/ml | -0.07 | 0.35 | 0.9 | 0.5 – 1.9 | 0.839 |
| | | | | | | **0.077* |
| NP non-haemolytic *Streptococcus* spp. | not isolated | referent | | 1.0 | | |
| | Isolated | -0.86 | 0.42 | 0.4 | 0.2 – 1.0 | 0.039 |
| | | | | | | **0.041* |
| NP *Actinobacillus/Pasteurella* spp. | not isolated | referent | | 1.0 | | |
| | Isolated | 0.74 | 0.36 | 2.1 | 1.0 – 4.3 | 0.039 |
| | | | | | | **0.038* |

*Likelihood ratio statistic $\chi^2$ P-value
NP = nasopharyngeal swab isolate
TW = tracheal wash isolate
Hosmer-Lemeshow $\chi^2$ = 7.10; P = 0.53

## 4.4 Cases *vs* 'subclinical' controls

### 4.4.1 Univariable analyses

Table 4.10 summarises univariable associations between clinically apparent respiratory disease and non-infectious explanatory variables using 328 'subclinical' controls. Using this set of controls, horses were at increased risk of being a clinical case with younger age, being female, increasing tracheal mucopus, presence of tracheal haemorrhage and less time since entering the training yard. Previous racing was marginally inversely associated with clinical disease.

**Table 4.10: Univariable associations between clinically apparent cases and non-infectious explanatory variables using 'subclinical' controls**

| *Explanatory variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Age group*** | Yearlings | 9.1 | 2.4 – 34.9 | 0.001 |
| | 2 year-olds | referent | | |
| | 3 year-olds | 0.6 | 0.3 – 0.9 | 0.024 |
| | ≥4 year-olds | 0.8 | 0.4 – 1.6 | 0.489 |
| ***Sex*** | Female | referent | | |
| | Male | 0.6 | 0.4 – 0.9 | 0.022 |
| ***Visible tracheal mucopus*** | none visible | referent | | |
| | slight | 3.0 | 1.7 – 5.0 | <0.001 |
| | moderate | 4.0 | 2.2 – 7.3 | <0.001 |
| | severe | 9.3 | 3.1 – 28.1 | <0.001 |
| ***Visible tracheal haemorrhage*** | none visible | referent | | |
| | visible | 4.5 | 1.5 – 13.3 | 0.006 |
| ***Time since entering training yard*** | <1 month | 3.8 | 1.7 – 8.4 | 0.001 |
| | 1-3 months | 2.8 | 1.5 – 5.1 | 0.001 |
| | >3 months | referent | | |
| ***Raced previously*** | No | referent | | |
| | Yes | 0.7 | 0.5 – 1.1 | 0.090 |
| ***Time since last race*** | Never raced | referent | | |
| | 1-7 days | 0.4 | 0.2 – 1.1 | 0.083 |
| | >7 days | 0.7 | 0.5 – 1.2 | 0.195 |

Table 4.11 shows that with univariable analysis, clinically apparent cases of respiratory disease compared to 'subclinical' controls were significantly associated with tracheal washes containing $>10^4$ cfu/ml total bacteria and non-haemolytic *Streptococcus* spp. and with $\log_{10}$ cfu/ml increases of *Pasteurella/Actinobacillus* spp. and *S. zooepidemicus.* The strengths of these associations were generally lower than for the analyses with 'healthy' controls or 'all' controls combined. Presence of *Acinetobacter* spp. and *Staphylococcus* spp. in tracheal washes were inversely associated with clinical disease. The presence of *S. pneumoniae* in tracheal washes was not associated with disease in this analysis. Both *M. felis* and NFGF *Mycoplasma* spp. present in tracheal washes were associated with clinical disease but *M. equirhinis* was not. There was no association between clinically apparent disease and viral infection or presence of any individual bacterial species in nasopharyngeal swabs.

## Table 4.11: Univariable associations between clinically apparent cases and microbiological explanatory variables using 'subclinical' controls

| *Explanatory variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Tracheal wash bacterial isolates*** | | | | |
| Total bacterial count | none isolated | referent | | |
| | $<10^2$ cfu/ml | 0.6 | 0.3 – 1.4 | 0.222 |
| | $10^2$-$10^3$ cfu/ml | 0.9 | 0.4 – 1.7 | 0.661 |
| | $10^3$-$10^4$ cfu/ml | 1.5 | 0.7 – 3.1 | 0.283 |
| | $10^4$-$10^5$ cfu/ml | 2.2 | 1.0 – 4.8 | 0.044 |
| | $10^5$-$10^6$ cfu/ml | 3.4 | 1.4 – 8.2 | 0.006 |
| | $>10^6$ cfu/ml | 4.0 | 1.6 – 10.4 | 0.004 |
| *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 1.3 | 1.1 – 1.4 | <0.001 |
| *Streptococcus zooepidemicus* | log cfu/ml$^{-1}$ | 1.3 | 1.1 – 1.4 | <0.001 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 1.3 | 0.7 – 2.4 | 0.420 |
| *Streptococcus equisimilis* | not isolated | referent | | |
| | isolated | 2.6 | 0.8 – 8.5 | 0.109 |
| Non-haemolytic *Streptococcus* spp. | $<10^3$ cfu/ml | referent | | |
| | $10^3$-$10^4$ cfu/ml | 1.2 | 0.7 – 1.9 | 0.551 |
| | $>10^4$ cfu/ml | 2.6 | 1.2 – 5.6 | 0.015 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 0.3 | 0.1 – 0.9 | 0.025 |
| | $>10^2$ cfu/ml | 0.8 | 0.2 – 2.7 | 0.675 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 0.9 | 0.4 – 2.0 | 0.717 |
| *Bordetella bronchiseptica* | not isolated | referent | | |
| | isolated | 0.9 | 0.1 – 10.9 | 0.953 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 1.3 | 0.2 – 10.1 | 0.792 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 0.7 | 0.3 – 1.8 | 0.489 |
| *Pseudomonas* spp. | not isolated | referent | | |
| | isolated | ∞ | – | – |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 0.5 | 0.05 – 4.5 | 0.527 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 0.3 | 0.2 – 0.6 | 0.001 |
| | $>10^2$ cfu/ml | 0.6 | 0.4 – 1.1 | 0.092 |
| ***Tracheal wash Mycoplasma isolates*** | | | | |
| *Mycoplasma felis* | not isolated | referent | | |
| | isolated | 4.0 | 1.2 – 13.0 | 0.024 |
| NFGF *Mycoplasma* spp. | not isolated | referent | | |
| | isolated | 4.5 | 1.2 – 16.9 | 0.025 |
| *Mycoplasma equirhinis* | not isolated | referent | | |
| | isolated | 0.9 | 0.5 – 1.6 | 0.631 |

NFGF = Non-felis glucose fermenting

Table 4.11 continued

| *Variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Serological evidence of viral infection*** | | | | |
| Equine herpesvirus | no seroconversion | referent | | |
| | seroconversion | 1.3 | 0.4 – 4.2 | 0.643 |
| Equine rhinovirus | no seroconversion | referent | | |
| | seroconversion | 1.6 | 0.4 – 5.9 | 0.493 |
| Adenovirus | no seroconversion | referent | | |
| | seroconversion | 1.6 | 0.1 – 17.5 | 0.722 |
| Influenza | no seroconversion | referent | | |
| | seroconversion | 4.0 | 0.5 – 29.2 | 0.174 |
| ***Nasopharyngeal swab bacterial isolates*** | | | | |
| *Pasteurella* spp. | not isolated | referent | | |
| | isolated | 1.2 | 0.7 – 2.1 | 0.522 |
| *Streptococcus zooepidemicus* | not isolated | referent | | |
| | isolated | 1.3 | 0.8 – 2.1 | 0.328 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 0.7 | 0.4 – 1.5 | 0.376 |
| Non-haemolytic *Streptococcus* spp. | not isolated | referent | | |
| | isolated | 0.6 | 0.3 – 1.2 | 0.141 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | isolated | 0.6 | 0.3 – 1.1 | 0.119 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 0.9 | 0.4 – 1.6 | 0.637 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 0.4 | 0.04 – 3.2 | 0.368 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 1.8 | 0.4 – 7.8 | 0.463 |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 0.8 | 0.1 – 8.9 | 0.835 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | isolated | 0.9 | 0.5 – 1.7 | 0.685 |

### 4.2.3 Multivariable analyses

Table 4.12 summarises the final multivariable CLR model for clinically apparent respiratory disease using only 'subclinical' controls, excluding the 2 matched sets in which influenza infections occurred. After controlling for microbiological variables, clinical respiratory disease remained significantly associated with age and time since entry into the training yard; with younger horses and those that had been in training less than one month at increased risk. Risk of being a case was significantly and positively associated with presence of *Actinobacillus/Pasteurella* spp. and *M. felis* in tracheal washes but was inversely associated with *Staphylococcus* and *Acinetobacter* spp. in washes. No statistically significant interactions were identified in this model.

**Table 4.12: Final multivariable CLR model for associations between clinically apparent respiratory disease and explanatory variables using 'subclinical' controls**

| *Explanatory variable* | | β | S.E.β | *Adjusted OR* | *95% CI* | *P-value* |
|---|---|---|---|---|---|---|
| Age group | Yearlings | 2.26 | 1.20 | 9.5 | 0.9 – 100 | 0.060 |
| | 2 year-olds | referent | | 1.0 | | |
| | 3 year-olds | -0.48 | 0.35 | 0.6 | 0.3 – 1.2 | 0.172 |
| | ≥4 year-olds | -0.17 | 0.50 | 0.8 | 0.3 – 2.2 | 0.732 |
| | | | | | | **0.089* |
| Time since entering training yard | <1 month | 1.32 | 0.62 | 3.8 | 1.1 – 12.7 | 0.033 |
| | 1-3 months | 0.62 | 0.42 | 1.9 | 0.8 – 4.2 | 0.134 |
| | >3 months | referent | | 1.0 | | |
| | | | | | | **0.065* |
| TW *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 0.16 | 0.08 | 1.2 | 1.0 – 1.4 | 0.050 |
| | | | | | | **0.047* |
| TW *Staphylococcus* spp. | not isolated | referent | | 1.0 | | |
| | <$10^2$ cfu/ml | -1.06 | 0.45 | 0.3 | 0.1 – 0.8 | 0.019 |
| | >$10^2$ cfu/ml | -0.09 | 0.30 | 0.9 | 0.5 – 1.6 | 0.755 |
| | | | | | | **0.041* |
| TW *Acinetobacter* spp. | not isolated | referent | | 1.0 | | |
| | <$10^2$ cfu/ml | -1.34 | 0.58 | 0.3 | 0.1 – 0.8 | 0.022 |
| | >$10^2$ cfu/ml | -0.23 | 0.69 | 0.8 | 0.2 – 3.1 | 0.739 |
| | | | | | | **0.038* |
| TW *M. felis* | not isolated | referent | | 1.0 | | |
| | Isolated | 1.30 | 0.68 | 3.7 | 1.0 – 13.8 | 0.056 |
| | | | | | | **0.051* |

*Likelihood ratio statistic $\chi^2$ P-value TW = tracheal wash isolate
Hosmer-Lemeshow $\chi^2$ = 4.86; P = 0.77

## 4.5 Subclinical cases *vs* 'healthy' controls

### 4.5.1 Univariable analyses

Table 4.13 summarises univariable associations between subclinical respiratory disease in controls with airway inflammation (cases in this analysis) and non-infectious explanatory variables using 'healthy' controls that had no evidence of airway inflammation. Using this definition of cases and controls, subclinical respiratory disease was not significantly associated with any of the non-infectious explanatory variables that were associated with clinically apparent respiratory disease.

**Table 4.13: Univariable associations between subclinical respiratory disease in controls and non-infectious explanatory variables using 'healthy' controls**

| *Explanatory variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Age group*** | Yearlings | 1.1 | 0.3 – 3.8 | 0.903 |
| | 2 year-olds | referent | | |
| | 3 year-olds | 0.8 | 0.5 – 1.2 | 0.807 |
| | ≥4 year-olds | 1.1 | 0.6 – 2.1 | 0.243 |
| ***Sex*** | Female | referent | | |
| | Male | 0.7 | 0.5 – 1.1 | 0.129 |
| ***Visible tracheal haemorrhage*** | none visible | referent | | |
| | visible | 1.4 | 0.4 – 5.3 | 0.624 |
| ***Time since entering training yard*** | <1 month | 2.1 | 0.8 – 5.4 | 0.123 |
| | 1-3 months | 1.4 | 0.8 – 2.6 | 0.285 |
| | >3 months | referent | | |
| ***Raced previously*** | No | referent | | |
| | Yes | 0.7 | 0.4 – 1.0 | 0.072 |
| ***Time since last race*** | Never raced | referent | | |
| | 1-7 days | 1.0 | 0.5 – 2.0 | 0.921 |
| | >7 days | 0.7 | 0.5 – 1.0 | 0.046 |

Table 4.14 shows that with univariable analysis, subclinical disease in controls compared to 'healthy' controls was significantly associated with increasing total bacterial count, *Pasteurella/Actinobacillus* spp. and *S. zooepidemicus* and $>10^4$ cfu/ml non-haemolytic *Streptococcus* spp. Presence of *S. pneumoniae and M. equirhinis* in tracheal washes were also associated with subclinical disease, whereas *M. felis* and NFGF *Mycoplasma* spp. were not. There was no association between subclinical disease and viral infection or presence of any individual bacterial species in nasopharyngeal swabs.

**Table 4.14: Univariable associations between subclinical respiratory disease in controls and microbiological explanatory variables using 'healthy' controls**

| *Explanatory variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Tracheal wash bacterial isolates*** | | | | |
| Total bacterial count | none isolated | referent | | |
| | $<10^2$ cfu/ml | 1.8 | 1.1 – 3.2 | 0.026 |
| | $10^2$-$10^3$ cfu/ml | 1.7 | 1.0 – 2.9 | 0.038 |
| | $10^3$-$10^4$ cfu/ml | 1.8 | 1.0 – 3.1 | 0.048 |
| | $10^4$-$10^5$ cfu/ml | 2.7 | 1.4 – 5.2 | 0.004 |
| | $10^5$-$10^6$ cfu/ml | 4.1 | 1.5 – 11.0 | 0.005 |
| | $>10^6$ cfu/ml | ∞ | – | – |
| *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 1.3 | 1.1 – 1.5 | 0.001 |
| *Streptococcus zooepidemicus* | log cfu/ml$^{-1}$ | 1.5 | 1.2 – 1.8 | <0.001 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 2.2 | 1.1 - 4.3 | 0.022 |
| *Streptococcus equisimilis* | not isolated | referent | | |
| | isolated | 0.9 | 0.3 - 3.2 | 0.859 |
| Non-haemolytic *Streptococcus* spp. | $<10^3$ cfu/ml | referent | | |
| | $10^3$-$10^4$ cfu/ml | 1.4 | 0.9 – 2.2 | 0.135 |
| | $>10^4$ cfu/ml | 3.7 | 1.1 – 12.3 | 0.032 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 2.0 | 0.9 – 4.1 | 0.077 |
| | $>10^2$ cfu/ml | 4.0 | 1.0 – 16.8 | 0.058 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 1.5 | 0.7 - 3.4 | 0.356 |
| *Bordetella bronchiseptica* | not isolated | referent | | |
| | isolated | 0.5 | 0.1 - 3.4 | 0.467 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 0.5 | 0.1 - 2.3 | 0.355 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 1.9 | 0.8 - 4.5 | 0.135 |
| *Pseudomonas* spp. | not isolated | referent | | |
| | isolated | 1/∞ | – | – |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 3.7 | 0.4 - 37.6 | 0.264 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | $<10^2$ cfu/ml | 1.0 | 0.6 – 1.6 | 0.991 |
| | $>10^2$ cfu/ml | 1.3 | 0.8 – 2.0 | 0.335 |
| ***Tracheal wash Mycoplasma isolates*** | | | | |
| *Mycoplasma felis* | not isolated | referent | | |
| | isolated | 2.9 | 0.3 - 25.8 | 0.349 |
| NFGF *Mycoplasma* spp. | not isolated | referent | | |
| | isolated | 0.7 | 0.2 - 2.7 | 0.566 |
| *Mycoplasma equirhinis* | not isolated | referent | | |
| | isolated | 2.5 | 1.4 - 4.6 | 0.004 |

NFGF = Non-felis glucose fermenting

Table 4.14 continued

| *Variable* | | *Crude OR* | *95% CI* | *P-value* |
|---|---|---|---|---|
| ***Serological evidence of viral infection*** | | | | |
| Equine herpesvirus | no seroconversion | referent | | |
| | seroconversion | 1.9 | 0.6 – 5.9 | 0.249 |
| Equine rhinovirus | no seroconversion | referent | | |
| | seroconversion | 1.8 | 0.4 – 7.6 | 0.404 |
| Adenovirus | no seroconversion | referent | | |
| | seroconversion | 1.3 | 0.2 – 8.4 | 0.792 |
| Influenza | no seroconversion | referent | | |
| | seroconversion | ∞ | – | – |
| ***Nasopharyngeal swab bacterial isolates*** | | | | |
| *Pasteurella* spp. | not isolated | referent | | |
| | isolated | 1.3 | 0.8 – 2.0 | 0.337 |
| *Streptococcus zooepidemicus* | not isolated | referent | | |
| | isolated | 1.2 | 0.8 - 1.9 | 0.277 |
| *Streptococcus pneumoniae* | not isolated | referent | | |
| | isolated | 1.3 | 0.8 - 2.4 | 0.302 |
| Non-haemolytic *Streptococcus* spp. | not isolated | referent | | |
| | isolated | 0.7 | 0.4 - 1.3 | 0.296 |
| *Acinetobacter* spp. | not isolated | referent | | |
| | isolated | 1.0 | 0.6 - 1.6 | 0.952 |
| *Bacillus* spp. | not isolated | referent | | |
| | isolated | 1.3 | 0.7 - 2.1 | 0.396 |
| *Enterobacter* spp. | not isolated | referent | | |
| | isolated | 1.2 | 0.3 - 4.2 | 0.807 |
| *Escherichia coli* | not isolated | referent | | |
| | isolated | 1.1 | 0.3 - 4.3 | 0.868 |
| *Serratia* spp. | not isolated | referent | | |
| | isolated | 0.6 | 0.1 - 3.3 | 0.522 |
| *Staphylococcus* spp. | not isolated | referent | | |
| | isolated | 1.0 | 0.6 - 1.7 | 0.985 |

### 4.5.2 Multivariable analyses

Table 4.15 summarises 2 final multivariable CLR models for subclinical respiratory disease compared with 'healthy' controls, excluding the 2 matched sets in which influenza infections occurred. After controlling for other variables, subclinical respiratory disease was only significantly associated with increasing *S. zooepidemicus* in tracheal washes. *S. pneumoniae* was forced into model 1 and *Actinobacillus/Pasteurella* spp. forced into model 2 as they each approached statistical significance (Wald P-values = 0.069 & 0.073, respectively), but once one of them had been included, then the other lost significance. In neither of these models was subclinical inflammatory airway disease associated with age or

time since entering training. No statistically significant interactions were identified in these models.

**Table 4.15: 2 final multivariable CLR models for associations between subclinical respiratory disease and explanatory variables using 'healthy' controls**

| *Explanatory variable* | | β | S.E.β | *Adjusted OR* | *95% CI* | *P-value* |
|---|---|---|---|---|---|---|
| *Model 1* | | | | | | |
| TW *S. zooepidemicus* | log cfu/ml$^{-1}$ | 0.35 | 0.09 | 1.4 | 1.2 – 1.7 | <0.001 |
| TW *S. pneumoniae* | not isolated | referent | | 1.0 | | |
| | Isolated | 0.68 | 0.37 | 2.0 | 0.9 – 4.1 | 0.069 |
| LRS $\chi^2$ P-value = 0.062, Hosmer-Lemeshow $\chi^2$ = 2.71; P = 0.95 | | | | | | |
| *Model 2* | | | | | | |
| TW *S. zooepidemicus* | log cfu/ml$^{-1}$ | 0.31 | 0.10 | 1.4 | 1.1 – 1.7 | 0.002 |
| TW *Pasteurella/Actinobacillus* spp. | log cfu/ml$^{-1}$ | 0.16 | 0.09 | 1.2 | 1.0 – 1.4 | 0.073 |
| LRS $\chi^2$ P-value = 0.070, Hosmer-Lemeshow $\chi^2$ = 4.73; P = 0.79 | | | | | | |

## 4.6 Post-fit diagnostics of final conditional logistic regression models

### 4.6.1 Goodness of fit of final models (Hosmer-Lemeshow statistics)

The observed and expected numbers of cases predicted by final multivariable conditional logistic regression models for the different case and control definitions in each decile of risk are summarised in Table 4.16. All final regression models demonstrated adequate goodness of fit as measured by non-significant Hosmer-Lemeshow statistics (Hosmer & Lemeshow, 1989). Although there are considerable limitations in the interpretation of non-statistically significant results in this sort of evaluation, results do confirm that there were no serious discrepancies between the fitted models and the distribution of cases in the study.

**Table 4.16: Observed and expected numbers of cases by decile of risk for each final multivariable conditional logistic regression analysis**

| *Model* | Decile of risk 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Cases vs 'all' controls* | | | | | | | | | | | |
| Observed cases (n) | 2 | 2 | 4 | 7 | 10 | 13 | 13 | 12 | 19 | 39 | 121 |
| Expected cases (n) | 1.2 | 3.2 | 4.9 | 6.7 | 8.5 | 10.4 | 12.7 | 15.4 | 19.9 | 38.1 | 121.0 |
| Total cases & controls (n) | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 65 | 596 |
| *Hosmer-Lemeshow $\chi^2$ statistic (8 d.f.) = 3.45; P = 0.90* | | | | | | | | | | | |
| *Cases vs 'healthy' controls* | | | | | | | | | | | |
| Observed cases (n) | 1 | 3 | 2 | 7 | 7 | 10 | 15 | 10 | 20 | 36 | 111 |
| Expected cases (n) | 0.8 | 2.6 | 4.0 | 5.3 | 6.8 | 9.3 | 12.0 | 15.3 | 20.5 | 34.3 | 110.9 |
| Total cases & controls (n) | 34 | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 39 | 353 |
| *Hosmer-Lemeshow $\chi^2$ statistic (8 d.f.) = 7.10; P = 0.53* | | | | | | | | | | | |
| *Cases vs 'subclinical' controls* | | | | | | | | | | | |
| Observed cases (n) | 2 | 3 | 6 | 10 | 11 | 11 | 10 | 14 | 21 | 28 | 116 |
| Expected cases (n) | 1.6 | 4.0 | 5.5 | 7.6 | 9.7 | 11.8 | 13.8 | 16.4 | 19.5 | 26.1 | 116.0 |
| Total cases & controls (n) | 36 | 36 | 36 | 36 | 36 | 36 | 36 | 36 | 36 | 35 | 359 |
| *Hosmer-Lemeshow $\chi^2$ statistic (8 d.f.) = 4.86; P = 0.77* | | | | | | | | | | | |
| *Subclinical cases vs 'healthy' controls: model 1 including S. pneumoniae* | | | | | | | | | | | |
| Observed cases (n) | 13 | 20 | 21 | 27 | 27 | 32 | 32 | 38 | 44 | 48 | 302 |
| Expected cases (n) | 12.9 | 17.9 | 21.1 | 24.7 | 27.1 | 31.9 | 36.1 | 40.1 | 43.8 | 46.3 | 301.9 |
| Total cases & controls (n) | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 54 | 585 |
| *Hosmer-Lemeshow $\chi^2$ statistic (8 d.f.) = 2.71; P = 0.95* | | | | | | | | | | | |
| *Subclinical cases vs 'healthy' controls: model 2 including Pasteurella/Actinobacillus spp.* | | | | | | | | | | | |
| Observed cases (n) | 10 | 20 | 20 | 26 | 32 | 28 | 33 | 41 | 45 | 47 | 302 |
| Expected cases (n) | 12.9 | 17.7 | 21.1 | 24.9 | 27.4 | 31.9 | 35.8 | 40.0 | 44.0 | 46.2 | 301.9 |
| Total cases & controls (n) | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 59 | 54 | 585 |
| *Hosmer-Lemeshow $\chi^2$ statistic (8 d.f.) = 4.73; P = 0.79* | | | | | | | | | | | |

### 4.6.2 Exclusion of observations with the largest delta-beta values

Examination of each final conditional logistic regression analysis model by sequential exclusion of observations with the largest delta-beta values i.e. those observations predicted to have the largest influence on the fit of models, demonstrated that all models were generally stable and robust. There was only one situation where the exclusion of a single

observation had a significant effect on the fit of a final model. Examination of observations with the largest delta-beta values for each model term showed that for the yearling category of AGECODE there was one observation (1659) with a very large delta-beta value and following its exclusion, models failed to converge. Closer examination of the data showed that this was because among the strata with yearling cases, observation 1659 was the only 2-year-old control i.e. member of the referent category. This was because of the small number of yearlings in the data (horses entered training in November and were re-classified as 2-year-olds the following January) such that exclusion of observation 1659 effectively meant that yearlings had an infinite OR due to the absence of any individuals in the referent group for the few relevant strata.

### 4.6.3 Examination of model residuals

For the largest final regression model using cases and 'all' controls (n=596) there were 99 observations with residuals with absolute values >0.5, which comprised 8 controls and 91 cases. Table 4.17 summarises the distribution of controls and cases with absolute residuals >0.5 between categories of the statistically significant variables retained in the final multivariable regression model. The distribution among categories of significantly retained variables indicated why the model most poorly predicts the clinical disease outcome status of these observations.

Among the 8 controls with high residual values, 3 were yearlings (the highest risk age group for clinical respiratory disease), 7 had entered training <3 months previously, 4 had $>10^3$ cfu/ml *Actinobacillus/Pasteurella* spp. in tracheal washes, 7 had no *Staphylococcus* or *Acinetobacter* spp. isolated from tracheal washes and one had *M. felis* isolated. Among the group of cases most poorly predicted by the model, all were 2-year-olds or older (i.e. not yearlings), 71 (78%) had entered training more than 3 month previously, 72 (79%) had $<10^3$ cfu/ml *Actinobacillus/Pasteurella* spp. in tracheal washes,

and 89 (98%) were negative for *M. felis* in their tracheal washes. These results indicate that a large proportion of the cases in this analysis were apparently not well predicted by the final model. This may be consistent with either i) the effects of factors that were not measured (e.g. stable dust or other unidentified infections), ii) the use of 3 clinical signs in the case definition provided a mixture of case types that actually had different risk factors or iii) although each of the risk factors identified were significantly associated with disease, only a small proportion of animals were positive for all of them.

**Table 4.17: Distribution of controls and cases with absolute residual values >0.5 between categories of significantly retained variables from the final multivariable regression model of cases vs 'all' controls**

| Risk factor | Category | Controls | | Cases | |
|---|---|---|---|---|---|
| | | n | % | n | % |
| Overall | | 8 | 100 | 91 | 100 |
| Age group | Yearlings | 3 | 37.5 | 0 | 0 |
| | 2 year-olds | 3 | 37.5 | 57 | 63 |
| | 3 year-olds | 2 | 25 | 24 | 26 |
| | ≥4 year-olds | 0 | 0 | 10 | 11 |
| Time since entering training yard | <1 month | 4 | 50 | 1 | 1 |
| | 1-3 months | 3 | 37.5 | 19 | 21 |
| | >3 months | 1 | 12.5 | 71 | 78 |
| TW *Actinobacillus/Pasteurella* spp. | none isolated | 4 | 50 | 56 | 62 |
| | $<10^2$ cfu/ml | 0 | 0 | 2 | 2 |
| | $10^2$-$10^3$ cfu/ml | 0 | 0 | 14 | 15 |
| | $10^3$-$10^4$ cfu/ml | 2 | 25 | 9 | 10 |
| | $10^4$-$10^5$ cfu/ml | 0 | 0 | 5 | 5 |
| | $10^5$-$10^6$ cfu/ml | 0 | 0 | 3 | 3 |
| | $>10^6$ cfu/ml | 2 | 25 | 2 | 2 |
| TW *Staphylococcus* spp. | None isolated | 7 | 87.5 | 58 | 64 |
| | $<10^2$ cfu/ml | 1 | 12.5 | 11 | 12 |
| | $>10^2$ cfu/ml | 0 | 0 | 22 | 24 |
| TW *Acinetobacter* spp. | None isolated | 7 | 87.5 | 83 | 91 |
| | $<10^2$ cfu/ml | 0 | 0 | 4 | 4 |
| | $>10^2$ cfu/ml | 1 | 12.5 | 4 | 4 |
| TW *M. felis* | None isolated | 7 | 87.5 | 89 | 98 |
| | Isolated | 1 | 12.5 | 2 | 2 |

# CHAPTER 5

# DISCUSSION

## 5.1 Discussion of multivariable analyses findings

Use of different case and control definitions in 4 separate analyses provided a better understanding of risk factors for this complex respiratory disease syndrome of young horses. There is probably a spectrum of severity of presentation of respiratory disease in horses and this includes subclinical inflammatory airway disease (IAD), which is not evidenced by any overt clinical signs, being diagnosed only with endoscopy and lavage of the trachea after exercise. This subclinical condition is being increasingly recognised in athletic young horses (Burrell, 1985; Wood *et al.*, 1993a; Burrell *et al.*, 1994; Moore *et al.*, 1995; Burrell *et al.*, 1996; Moore, 1996; Christley *et al.*, 1999b; Chapman *et al.*, 2000; Christley *et al.*, 2001a; 2001b) and so an important and unique aspect of this study was the consideration of the occurrence and possible influence of subclinical IAD on the risk factors for clinically apparent respiratory disease. The findings of the multivariable analyses for cases presenting clinically were therefore compared with separate healthy and subclinical control subgroups in the first instance and then the factors accounting for differences between healthy and subclinical controls were considered. Finally, the risk factors for clinically apparent respiratory disease that arose in an overall large model using 'all' controls together, irrespective of IAD status, were considered in light of the findings from the 3 smaller models.

### 5.1.1 Cases *vs* 'healthy' controls

Controls in this analysis did not have signs of IAD as defined by endoscopic and cytological parameters (Whitwell & Greet, 1984; Burrell *et al.*, 1996). However, in contrast

to previous studies (Burrell *et al.*, 1996; Wood, 1999) the definition of controls here was that these horses had inflammation scores of zero out of 3 – so a very stringent definition of respiratory health was used. Multivariable analysis showed that after controlling for other factors, clinical respiratory disease was significantly associated with younger age and recent entry into the racing yard. This was consistent with, but not proof of, an infectious aetiology as would be expected with acquisition of immunity against infection in young horses or recent arrivals experiencing novel infectious challenge. In the event that horses entered training at exactly the same time e.g. as yearlings, then these variables would have measured the same thing. However, as there was a wide variation in the time since entry into training within each 'Thoroughbred' age category, it is more likely that there was an overlap between these variables. After controlling for these factors, horses were at increased risk of clinical disease with recovery of *Actinobacillus/Pasteurella* spp. and *S. zooepidemicus* from tracheal washes and *Actinobacillus/Pasteurella* spp. from nasopharyngeal swabs. There was a significant inverse association found with low numbers ($<10^2$ cfu/ml) of *Staphylococcus* spp. in washes and non-haemolytic *Streptococcus* spp. in swabs. This may have reflected the dynamic nature of colonisation by micro-organisms in the respiratory tract, in that as the more pathogenic bacteria such as *Actinobacillus/Pasteurella* spp and *S. zooepidemicus* increased in number they replaced transient or commensal bacteria such as *Staphylococcus* spp. and non-haemolytic *Streptococcus* spp.. However, although culture techniques were designed to attempt to detect species of bacteria even in small numbers by use of different selective media, it was still possible that in certain circumstances small numbers of transient or commensal bacteria from all horses may have been masked by larger numbers of other bacteria growing on the same media when large numbers of others overgrew them. So whether these findings represent a true phenomenon is unknown but worthy of further study. It is of note that when *Actinobacillus/Pasteurella* spp. were found in both the trachea and nasopharynx they were associated with disease without apparently confounding each

other. The issue of contamination of tracheal samples collected via an endoscope passed through the upper respiratory tract is discussed in further detail in Section 5.2.4.

### 5.1.2 Cases *vs* 'subclinical' controls

Controls in this analysis were defined on the basis of having signs of IAD based on endoscopic and cytological parameters. Importantly, in contrast with earlier AHT studies in which horses with inflammation scores of one were defined as non-diseased controls (Burrell *et al.*, 1996; Wood, 1999), in this thesis outwardly healthy horses with inflammation scores of one or greater out of 3 were defined as 'subclinically' affected controls. Multivariable analysis using 'subclinical' controls showed that, after controlling for the effects of other variables, clinical disease was still significantly associated with younger age, recent entry into the yard, presence of *Actinobacillus/Pasteurella* spp. in tracheal wash samples and relative absence of small numbers of *Staphylococcus* spp. In addition, horses were at increased risk of clinically apparent disease with the isolation of *M. felis* from tracheal washes but at decreased risk with isolation of small numbers of *Acinetobacter* spp. in washes. In contrast to the analysis with 'healthy' controls (i.e. no pathology detected on endoscopy and tracheal wash), *S. zooepidemicus* in tracheal washes and nasopharyngeal bacterial factors were not significantly associated with clinical respiratory disease.

### 5.1.3 Subclinical cases *vs* 'healthy' controls

Cases in this analysis were taken as those horses that had been defined as 'subclinical' controls in the previous analysis and they were compared with horses that were selected as 'healthy' controls. Both groups of horses had been selected as controls for the main study and were later subdivided according to endoscopic and cytological evidence of IAD; neither group had outward signs of respiratory disease at the time of sampling. Multivariable analysis conducted with the same criteria as previous analyses showed that all

other variables were confounded by the presence of *S. zooepidemicus* in tracheal washes. There was also an apparently increased risk with *S. pneumoniae* or *Actinobacillus/Pasteurella* spp. isolated from tracheal washes having controlled for *S. zooepidemicus*. These infections, which were forced into 2 final models as their presence after controlling for *S. zooepidemicus*, were nearly significant at the 5% level (Wald $\chi^2$ P values = 0.069 and 0.073, respectively and LRS $\chi^2$ P values = 0.062 and 0.07, respectively).

In contrast to the analyses using clinically apparent respiratory disease cases, 'subclinical' disease was not significantly associated with younger age or time since entry into the yard. This suggests that horses may have at least slight airway inflammation, irrespective of their age and time in training, and would be consistent with immunity that increases with age and time in training but which is not complete or with an underlying problem that is largely unrelated to infection. This finding should be considered in the context that horses with an inflammation score of one out of 3 (probably indicating only slight airway inflammation) were allocated to the 'subclinical' case/control group in this study.

In order to investigate whether any factors were associated with 'subclinical' disease among controls with inflammation scores of one, and because this level of inflammation had arbitrarily been considered 'normal' in previous studies (Burrell *et al.*, 1996; Wood, 1999), additional *post hoc* analyses were conducted. Analyses were performed comparing horses with inflammation scores of one with 'healthy' controls (zero inflammation scores, n=304), after exclusion of all controls with inflammation scores of 2 or 3.

Of 328 horses with evidence of 'subclinical' respiratory disease, 238 (73%) of these had an inflammation score of one out of a possible score of 3. These 238 animals were assigned a single inflammation score on the basis of i) moderate or severe visible mucopus in eight animals (3.4%), ii) ≥1000 nucleated cells/ml tracheal wash in 14 horses (5.9%) and iii) moderate or predominant amounts of tracheal wash neutrophils in 216 controls (90.7%). Results indicated that compared with horses with inflammation scores of zero, 'subclinical'

disease as evidenced by an inflammation score of one out of 3 was significantly associated with being female and the presence of *S. zooepidemicus* in tracheal washes.

### 5.1.4 Clinically apparent cases *vs* 'all' controls

Having considered 3 previous analyses with more specific definitions of cases and controls, the results of the main analysis used clinically apparent cases compared with 'all' controls, irrespective of their IAD status. In this multivariable CLR model clinical disease was significantly associated with age group, time since entry into the yard, *Actinobacillus/Pasteurella* spp. and *M. felis* isolated in tracheal washes and inversely with low numbers of *Staphylococcus* and *Acinetobacter* spp. in washes. Tracheal *S. zooepidemicus* and nasopharyngeal swab bacterial variables were not significantly associated with clinical respiratory disease in this model, although they had been associated with clinical disease in the analysis using 'healthy' controls.

It is clear that the final model using 'all' controls (i.e. 'healthy' and 'subclinical' controls together), when considered in relation to each of the separate models, is likely to reflect a composite of factors from these other models. This is most clearly demonstrated by infection with *S. zooepidemicus*, which was shown to be associated with both clinical and 'subclinical' disease, including disease defined by an inflammation score of one, when each was compared with only 'healthy' controls but was absent from the all and 'subclinical' only control models. This was because *S. zooepidemicus* in tracheal washes was significantly associated with 'subclinical' disease, evidenced predominantly by tracheal neutrophilia, among the controls as well as being associated with clinical disease among cases. Overall, there was no apparent significant net effect from *S. zooepidemicus* in favour of clinically apparent respiratory disease when horses with 'subclinical' IAD were used as or included in the control group. Similarly the effects of nasopharyngeal bacterial isolates of *Actinobacillus/Pasteurella* spp. and non-haemolytic *Streptococcus* spp. that were

associated with clinical disease compared with 'healthy' controls were not retained in the final model. In contrast, *Actinobacillus/Pasteurella* spp. in tracheal washes, although nearly significantly associated with 'subclinical' disease among controls, retained a significant association with clinically apparent respiratory disease when compared with only 'subclinical' controls as well as 'all' controls considered together. In multivariable analyses *M. felis* in tracheal washes was associated with clinical disease compared to 'subclinical' but not 'healthy' controls and this significant association was retained in the overall model with 'all' controls. Age, time since entry into the training yard and *Staphylococcus* spp. in low numbers in tracheal washes were significantly inversely associated with clinical but not 'subclinical' disease in these analyses.

Examination of observations with residual values >0.5 in the final main analysis (i.e. cases and 'all' controls) indicated that a large proportion of the cases in this analysis were apparently not well predicted by the variables included in the final model. The distribution of numbers of these cases between categories of retained variables showed that this was because large proportions of these cases were not yearlings and/or had been in training >3 months and/or did not have $>10^3$ cfu/ml *Actinobacillus/Pasteurella* spp. and/or *M felis* isolated from their tracheal washes. This suggests that there are other significant factors associated with respiratory signs in these cases that were not included in the final model. It is possible that there were effects from other unidentified infectious agents or from non-infectious factors such as stable dust, which is a recognised factor in triggering respiratory signs, particularly in older animals. In the absence of appropriate assays it is always possible that there are infections that remain undetected, the outbreak of *M. felis*-associated respiratory disease described by Wood *et al.* (1997a) illustrated this. The use of matching by trainer in this study was intended to help control for the immeasurable effects of such non-infectious factors as stable dust. However, we would expect inevitable variation of factors such as the effect of stable dust within training yards and particularly between individual

horses of different ages. There may also have been inadequate account taken of the effect of airway inflammation in the horses in this analysis. Although 'all' controls included animals with and without airway inflammation and it was known that cases had a much higher proportion of horses with inflammation scores ≥2 compared to controls, the final model did not include any specific factors (other than infections associated with IAD) to account for differences in the severity of airway inflammation between cases and controls. Related to this there may have been some additional influence from the exclusion of *S. zooepidemicus* infection from this analysis as $>10^3$ cfu/ml of *S. zooepidemicus* in tracheal washes was more prevalent in cases than controls and may have been the only infection identified in some cases. There may also have been problems related to the case definition used in this study in that horses were defined as a case if they presented with one or a combination of 3 signs (cough, nasal discharge, pyrexia). This may have made prediction more difficult if different signs were related to different factors.

### 5.1.5 Conclusions

Taken together the analyses conducted in this study using different case and control definitions provide considerable evidence that bacterial infections of the trachea, sometimes in conjunction with other factors, are predominantly associated with a range of presentations of respiratory disease in young horses in training. The milder end of the disease spectrum includes slight inflammation attributed predominantly to tracheal neutrophilia (inflammation score 1/3) in the absence of overt clinical signs and detected only by cytology of tracheal wash samples. The more severe end of the spectrum includes obvious signs of disease such as coughing, nasal discharge and pyrexia and this is associated with marked airway inflammation (inflammation score ≥2/3) as evidenced by visible tracheal mucopus, neutrophilia and high nucleated cell count. The study identified that tracheal infection with *S. zooepidemicus* was associated with an increased risk of clinically apparent

as well as subclinical respiratory disease when compared with 'healthy' horses that had no clinical, endoscopic or cytological evidence of disease (inflammation score 0/3). This remained true for horses that had a cytologically detectable airway neutrophilia but were otherwise considered to have a healthy respiratory tract. There was evidence that tracheal infections with *Actinobacillus/Pasteurella* spp. and *M. felis* were associated with clinically more apparent disease and that younger horses and those that had arrived more recently in the yards were at increased risk of clinical disease.

## 5.2 Current study findings in the context of existing knowledge

The current study is one of a few conducted by separate research groups in recent years that are starting to contribute to a better understanding of the epidemiology of respiratory disease in young horses. There have to date been relatively few studies conducted to investigate the association between respiratory disease in horses and a wide range of possible infectious agents, including viruses, bacteria and mycoplasmas and other non-infectious factors. It is recognised that the true biological credibility of the conclusions of individual studies is enhanced when they demonstrate broadly similar findings to other separate studies. This was referred to as 'consistency of association' by Bradford Hill (1965) in his criteria for assessing causality. The findings of this study are discussed particularly in the context of similar racehorse studies conducted in Sydney, Australia (Christley *et al.*, 1999b; 2001a; 2001b) and by 2 groups in the United Kingdom (Burrell, 1985; Wood *et al.*, 1993a; Burrell *et al.*, 1994; 1996; Chapman *et al.*, 2000). Where relevant, findings are also compared with work conducted on infectious causes of inflammatory respiratory disease in foals by Andy Hoffman (Hoffman *et al.*, 1993a; 1993c).

### 5.2.1 The effects of age and sex

The current study conducted in young flat-trained Thoroughbred racehorses is in general agreement with previous studies in demonstrating that the risk of clinically apparent respiratory disease decreased with increasing age in horses. In the current study the inverse association with age remained even after controlling for other factors including infections and time since entering training.

The AHT group has shown previously that the risk of infection with *S. zooepidemicus, S. pneumoniae* and *Pasteurella* spp. associated with airway inflammation was statistically significantly lower in horses 4 years or older compared with 3-year-olds and younger (Wood *et al.*, 1993a). Similarly, in 2 longitudinal studies, the risk of inflammatory airway disease associated with bacterial infection, particularly *S. zooepidemicus*, decreased with age (Burrell *et al.*, 1996; Wood, 1999). The prevalence of several bacterial infections and different disease presentations, including inflammatory lower airway disease, also decreased with increasing age in 2 recent studies (Wood *et al.*, 1998; Wood, 1999; Chapman *et al.*, 2000). The longitudinal study by Wood *et al.* (1998) also demonstrated that the incidence of specific infections decreased between 2 and 4 years of age and this was particularly marked for *S. pneumoniae*. A recent case control study conducted in Thoroughbred racehorses in Sydney, Australia also showed that the risk of coughing decreased significantly with increasing age, with yearlings and 2-year-olds being at greatest risk (Christley *et al.*, 1999b; 2001a; 2001b).

These multiple studies all demonstrate similar inverse associations between risk of infectious inflammatory respiratory disease and increasing age. This is most likely explained by immunity increasing with age in young horses with increased exposure to a wide range of species and different subtypes of organisms. This situation is analogous to that of young children acquiring immunity to common childhood infections during their early nursery/school years. It has been suggested that this time dependent (age and time in

training) decrease in clinically apparent respiratory disease, particularly coughing, may be due to an induced tolerance to airborne irritants, such as endotoxin (Christley *et al.*, 1999b; 2001a). It is likely that because endotoxin (lipopolysaccharide) is an integral part of gram negative bacteria such as *Pasteurella* spp., then it probably does play a role in the inflammatory processes seen in clinical infectious lower airway disease in young horses. To date several studies have demonstrated short-term tolerance to endotoxin in horses (Allen *et al.*, 1996; Barton *et al.*, 1996). In other species this phenomenon has been shown to lead to moderation of inflammatory processes such as reduction of the activation and recruitment of neutrophils and production of chemokines and cytokines (Shimada *et al.*, 2000). It is not clear at the moment, however, whether endotoxin tolerance can be maintained for the time scales (i.e. months) over which signs of respiratory disease persist before reducing in the horse (Wood, 1999). Further detailed studies are obviously required to better understand the exact role of bacterial as well as environmental endotoxin in clinical respiratory disease syndrome in young horses.

To date very few studies have considered the effect of gender or sex on risk of respiratory disease in horses. In the Australian study of risk factors for coughing, gender was not found to be significantly associated with disease after controlling for other factors, although at a univariable level colts and females were at apparently increased risk compared to geldings (Christley *et al.*, 1999b). In the larger current study, although differentiation between colts and geldings was not made and male horses at the univariate level appeared at decreased risk of clinically apparent respiratory disease compared to females, sex was not a significant risk factor in the final models.

### 5.2.2 The effects of training & racing

The simultaneous examination of the effects of previous racing and stage of training with other factors including age and infections, have not been reported previously, although

Christley *et al.* (1999b; 2001a) did examine recent racing and stage of training among non-infectious risk factors for coughing in Australian Thoroughbred racehorses. In the Australian study, multivariable analysis showed that the risk of coughing decreased with stage of training but horses that had raced within the last week were approximately 11 times more likely to cough than those that had never raced previously (Christley *et al.*, 1999b; 2001a). The variable 'time since entering training yard' used in the current UK study may be considered analogous to 'stage of training' used by Christley *et al.* because horses that had more recently entered training would be automatically at an earlier stage of training than those that had been in training longer. In agreement with Christley *et al.* (1999b; 2001a), this study found that horses that had entered training more recently were at significantly increased risk of clinically apparent respiratory disease, even after controlling for the effects of age and other factors. A study of both flat and national hunt racehorses in the UK also showed that 4-year-old flat trained horses were at significantly lower risk of airway inflammation than the same aged horses in national hunt yards (Chapman *et al.*, 2000). The difference between the 2 types of horses was most likely related to the time that they had been in training and, therefore, the time they had been encountering infections and developing immunity. The 4-year-old flat horses had generally been in training for at least 2 years and were much more likely to have developed immunity to infections. In contrast the 4-year-old NH horses would include horses from the flat and NH stores the latter having only just entered training and would be more likely to be suffering respiratory infections for the first time. The current data, when examined at a univariable level, showed that horses that had never raced previously were at increased risk of clinical respiratory disease compared with those that had raced before. However, when other factors including age, time in training and infections were simultaneously considered, racing at any time previously or within the last week were not found to be associated with clinical disease. The univariable effect had probably been confounded by these other factors. This contrasts with

the findings of Christley *et al.* (1999b; 2001a), where controlling of confounding resulted in a highly significant association between recent racing and the occurrence of coughing.

Differences in the amount of inhaled debris experienced by horses during racing has previously been proposed as a cause for markedly different rates of pleuropneumonia in American and British racing Thoroughbreds (Mair & Lane, 1989; Chaffin & Carter, 1993; Austin *et al.*, 1995; Raidal, 1995; Raidal *et al.*, 1997b). That is that dirt tracks, which are commonly used in North America, produce more inhaled debris than turf tracks that are more widely used in the UK and may be watered. Therefore, any fundamental differences in the commonly used racing surfaces in the respective countries may have accounted for this difference. Although horses in Sydney train predominantly on a non-turf surface, they do race on turf. The nearest track surfaces to dirt in the UK are the sand and petroleum based 'all-weather' surfaces, which are recognised as having increased 'kickback' compared with turf. The younger horses in the yards we studied did not generally use this type of surface in the early stages of their racing careers. Indeed, the only 2-year-old horse that was raced on such a surface in this study was a clinical case that showed signs within one week of racing on an 'all-weather' track. However, examination of horses that recently raced did not demonstrate an increased risk of clinical respiratory disease among the few horses (n=10) that had raced on an all-weather surfaces in the past week compared with those that had raced on turf in this study. Further work is obviously required to establish whether racing on all-weather surfaces is associated with an increased risk of clinically apparent respiratory disease in UK racehorses and whether this differs compared to other countries.

### 5.2.3 The effect of bacterial and mycoplasma infections of the trachea

As with previous studies of different forms of respiratory disease in young horses this study provides evidence for an association between *S. zooepidemicus* and *Actinobacillus/Pasteurella* spp. and clinically apparent respiratory disease (Burrell, 1985;

Wood *et al.*, 1993a; Burrell *et al.*, 1994; 1996; Christley *et al.*, 1999b; Chapman *et al.*, 2000; Christley *et al.*, 2001b). An important difference between this study and others previously reported is that many factors, including non-infectious as well as infectious variables were considered simultaneously for their association with clinical respiratory disease. In addition, the possible effect of subclinical inflammatory airway disease (IAD) was also considered and controlled for in examination of the data. This study was consistent with earlier findings of associations between bacterial infections and pyrexia (Burrell *et al.*, 1994) and coughing (Christley *et al.*, 1999b; 2001a; 2001b) both of which were included in the case definition used here. The study also confirmed the close association between non-specific signs of clinical respiratory disease (pyrexia, nasal discharge and coughing) and the likely presence of inflammatory airway disease (IAD) (Burrell *et al.*, 1996) and bacterial lower airway infection (Wood *et al.*, 1993a; 1998; Wood, 1999; Chapman *et al.*, 2000).

This study demonstrated that the group of organisms referred to as *Actinobacillus/Pasteurella* spp. were significantly associated with clinically apparent respiratory disease when compared with any classification of controls, including those with subclinical airway inflammation. Comparison of 'subclinical' with 'healthy' controls also showed that infection with this group of organisms was almost statistically significantly associated with subclinical IAD. Together this suggests that not only might *Actinobacillus/Pasteurella* spp. contribute to subclinical IAD but they might also have a role in contributing to clinically apparent respiratory disease. The association with clinically apparent disease was more robust for *Actinobacillus/Pasteurella* spp. than for *S. zooepidemicus* and might suggest a relatively more important role in clinical disease for some members of this group of organisms. Previous work on the *Pasteurella/Actinobacillus* spp. group has shown that specific identification of these species by colony morphology alone is difficult because of the similarity between them (Ward *et al.*, 1998). Accurate identification of species within the group requires study of detailed fermentation reactions to

different sugars but this is complicated, expensive and too time-consuming to carry out on large numbers of diagnostic samples. It is likely that grouping of these bacterial species resulted in an underestimation of the true extent of the effect of several specific pathogenic species within the group (such as *A. equuli, A. suis* and *A. lignieresii*), although some of the more pathogenic bacteria may have been uncommon (*A. suis* and *A. lignieresii*) (Ward *et al.*, 1998). Further detailed epidemiological studies are required to elucidate which species in the *Pasteurella/Actinobacillus* spp. group are associated with clinical presentation of respiratory disease in horses.

*Streptococcus zooepidemicus* is an important equine pathogen and is commonly associated with pneumonia, 'shipping fever' and inflammatory airway disease in young horses (Chanter, 1997). Detailed studies have shown that respiratory infections are common in weaned foals (Hoffman *et al.*, 1993a; 1993c) and young Thoroughbred racehorses (Wood *et al.*, 1993a) and that they are frequently protracted and recurrent. Age-specific decreases in incidence and prevalence of specific bacterial lower respiratory tract infections in racehorses, including *S. zooepidemicus*, have been demonstrated (Wood *et al.*, 1998) and this is consistent with gradual development of immunity. The exact reasons for this, however, are not known but may be due to cumulative and sequential infections (clonal succession) with non-cross immunising subtypes of *S. zooepidemicus*. In support of this there is evidence that equine sera can be highly discriminating in their ability to opsonise *S. zooepidemicus* isolates (Causey *et al.*, 1995) and which suggests that there are antigenic and immunogenic differences between strains of this bacterium. Therefore specific subtypes of *S. zooepidemicus* may be found to be particularly associated with respiratory disease in horses and this may also have resulted in an underestimation of the overall association of this species in this study.

Previous studies have shown significant association between *S. pneumoniae* tracheal infection and equine upper and lower respiratory tract disease, particularly in young horses

(Burrell *et al.*, 1986b; Mackintosh *et al.*, 1988) and experimental tracheal inoculation has reproduced disease (Blunden *et al.*, 1994). A statistically significant univariable association between infection with *S. pneumoniae* and both clinically apparent disease and subclinical IAD was also identified in this study and there was almost a significant association demonstrable with subclinical IAD when tracheal wash *S. pneumoniae* was included with tracheal wash *S. zooepidemicus* in a multivariable analysis. The failure to achieve statistical significance in multivariable analyses most likely reflected the low power of this study due to the low prevalence of *S. pneumoniae* compared to other infections. Equine *S. pneumoniae* isolates have to date all been of the same capsule type (type 3) (Mackintosh *et al.*, 1988). The marked reduction in risk of disease from *S. pneumoniae* with increasing age in young horses (Wood *et al.*, 1998; Wood, 1999) may be due to the rapid acquisition of immunity against this single capsule type. This appears to be in contrast to the situation with *S. zooepidemicus* and *Pasteurella/Actinobacillus* spp. for which solid immunity may be acquired only following serial infections by several non cross-protective subtypes (*S. zooepidemicus*) or even species (*Actinobacillus/Pasteurella*) (Wood *et al.*, 1993a; Wood, 1999).

In the current study multivariable analyses showed that *M. felis* was statistically significantly associated with clinical disease when compared with 'all' controls as well as only controls with subclinical IAD. However, at the univariable level tracheal *M. felis* infection was significantly associated with clinical respiratory disease when compared with each classification of controls (i.e. all, 'healthy' and 'subclinical') but was not associated with subclinical IAD compared with 'healthy' controls. Controlling for the combination of other factors, particularly tracheal *S. zooepidemicus* infection, probably accounted for why *M. felis* in the trachea did not remain significantly associated with clinical disease at the multivariable level in the model using 'healthy' controls as the comparison group. There was no association identified between clinical disease and tracheal isolation of other species

of mycoplasma at the multivariable level including *M. equirhinis*. However, non-felis glucose fermenting *Mycoplasma* spp. was significantly associated with clinically apparent respiratory disease at the univariable level. These results are in agreement with earlier reports of the potential for *M. felis* to produce clinical signs of respiratory disease including pleuritis (Ogilvie *et al.*, 1983; Rosendal *et al.*, 1986; Hoffman *et al.*, 1992a), pericarditis (Morley *et al.*, 1996) and lower respiratory tract inflammatory disease (Wood *et al.*, 1997a).

From this and other studies, there is a significant and strong association between presence of certain presumptively pathogenic species of bacteria, particularly *S. zooepidemicus, Actinobacillus/Pasteurella* spp. and *M. felis* and a range of severity of IAD in horses, as measured by inflammation score and clinical signs (Wood *et al.*, 1993a; Burrell *et al.*, 1994; Chapman *et al.*, 2000). Therefore, an additional *post hoc* analysis was conducted to investigate the nature of the association between tracheal infection with these bacterial species and different classifications of respiratory disease. An analysis was performed to investigate a possible linear trend in proportions of tracheal washes positive for any of these bacterial species across disease categories ordered according to increasing severity by chi-square test for linear trend under the STATCALC command in Epi-Info 6 (Dean *et al.*, 1994).

Tracheal washes were classified as positive if either *S. zooepidemicus, Actinobacillus/Pasteurella* spp. or *M. felis* were isolated from them and negative if none of the 3 bacterial species was recovered. As not all washes were examined for mycoplasma, those that were negative for *S. zooepidemicus* and *Actinobacillus/Pasteurella* spp. and were not submitted for mycoplasma culture were also classified as negative (rather than missing) in order to maximise sample size. Categories of disease outcome were classified and ordered according to inflammation score (0, 1 and ≥2 out of possible maximum of 3) in the first instance and these levels were subdivided according to whether horses had been

recruited to the study as cases or controls. Hence, disease categories were logically ordered from healthy controls with zero inflammation score as the baseline referent group, through to clinical cases with inflammation score of 2 or greater at the highest disease level (Table 5.1). Analysis output provided chi square value for linear trend, corresponding probability value as well as ORs as a measure of risk of a positive outcome at each disease level relative to the baseline group.

Results are summarised in Table 5.1 and showed that there was a highly statistically significant linear trend for increasing proportions (and hence trend of increasing ORs) of tracheal washes positive for *S. zooepidemicus, Actinobacillus/Pasteurella* spp. or *M. felis* as inflammation score increased. In addition, for horses with a given inflammation score, those presenting with clinical signs of respiratory disease (cases) had an increased proportion of positive washes and OR compared with controls not demonstrating overt clinical signs but with the same level of inflammation.

These data apparently confirm that inflammation score is an appropriate means of classifying respiratory disease status with respect to likely tracheal bacterial infection with presumptive pathogens. However, they also highlight that there was a moderate proportion of horses (24 of 157 cases (15%) that had tracheal washes examined for bacteria) that were classified as clinical cases but in which there was no evidence of tracheal inflammation or infection with *S. zooepidemicus, Actinobacillus/Pasteurella* spp. or *M. felis.* It is possible that other early stage bacterial, viral or mycoplasma infections were responsible for some of these cases, particularly prior to the development of marked inflammatory responses or that there was possibly some misclassification of cases or that there were other causes.

**Table 5.1: Proportions of tracheal washes (TWs) positive for *S. zooepidemicus*, *Actinobacillus/Pasteurella* spp. or *M. felis** among horses ordered according to inflammation score and presence (cases) or absence (controls) of clinical signs**

| Case/Control | Inflammation score | TWs (%) positive for bacterial spp.* | Total TWs examined (100%) | Odds Ratio |
|---|---|---|---|---|
| Control | 0 | 91 (30%) | 304 | 1.00 (ref) |
| Case | 0 | 18 (43%) | 42 | 1.76 |
| Control | 1 | 88 (37%) | 238 | 1.37 |
| Case | 1 | 25 (51%) | 49 | 2.44 |
| Control | ≥2 | 71 (79%) | 90 | 8.75 |
| Case | ≥2 | 53 (80%) | 66 | 9.54 |

$\chi^2$ for linear trend = 92.43 ($P \leq 0.00001$)

In this and similar previous studies tracheal washes were collected transendoscopically, which means that there was the potential for transfer of bacteria on the endoscope between consecutively sampled animals. However, all reasonable and practical precautions were taken to thoroughly disinfect the endoscope by immersion between animals, with disinfectant freshly made up between batches of animals. Although not specifically recorded for data analyses, it would be possible to examine the effect of the order in which animals were sampled on the quantitative bacteriology of tracheal wash samples to see whether horses sampled later tended to have higher bacterial counts due to cross contamination from earlier samplings due to inadequate disinfection between horses.

Although quantitative bacteriological techniques were applied in this study, for analytical purposes these were rounded down to the nearest whole $\log_{10}$ colony forming unit count and depending on their relative frequency these were subsequently treated as continuous, ordered categorical or even binary outcomes. This rounding and categorisation of bacterial counts meant that there was a loss of precision of colony count estimation, which would have inevitably lead to reductions in the significance of any trends in association, particulalry where infections were relatively rare and were consequently treated as binary outcomes.

### 5.2.4 The effect of nasopharyngeal bacterial infections

There have been several studies to evaluate the relative benefits of transtracheal aspiration and endoscopy for collection of distal tracheal samples (Sweeney *et al.*, 1989; Darien *et al.*, 1990; Christley *et al.*, 1999a). It is generally considered that whereas endoscopy does increase the likelihood of contamination of tracheal samples with bacteria of URT origin, a large proportion of non-contaminated samples can be obtained using this method (Wood *et al.*, 1993a). In addition endoscopy has the advantage that it can be readily repeated, does not require sedation in the majority of animals and has a much lower risk of serious adverse effects than the invasive transtracheal technique. To this end, tracheal endoscopy is still considered the technique of choice for obtaining post exercise tracheal wash samples from valuable Thoroughbred racehorses, that are undergoing routine respiratory health monitoring and which may or may not have subclinical IAD. The influence of different catheter designs on degree of oropharyngeal contamination during endoscopic collection of tracheal wash samples has not been directly assessed, although several studies have been conducted comparing endoscopic collection of tracheal samples with transtracheal aspiration, considered to be the 'gold standard' with respect to representative bacterial sampling as it bypasses contamination from the upper respiratory tract (Darien *et al.*, 1990; Christley *et al.*, 1999a). It is possible, however, that differences in the design of sterile catheters preplaced in the biopsy channel of the endoscope but withdrawn several inches from the end of the endoscope during introduction to the distal trachea, may not make a significant difference to the degree of oropharyngeal contamination. It is likely that contamination will occur to the outside of the end of the endoscope, irrespective of the design of catheter shielded in the biopsy channel. The degree of contamination is most likely to be dictated by the time spent negotiating the upper respiratory tract, particularly the pharynx and the density of pharyngeal infection, which is likely to vary between cases and controls. Cases with large numbers of LRT bacteria that

are coughing or have a nasal discharge would produce more URT contamination than controls that have smaller LRT bacterial numbers.

To investigate whether oropharyngeal/URT contamination was likely to be responsible for the presence of specific bacterial species in tracheal washes taken during the same sampling session and was, therefore, the explanation for the association between disease and tracheal bacterial infection, further *post hoc* analyses were conducted. To do this pathogen-specific analyses were conducted on data that were restricted to only those horses for which the specific bacteria had been recovered from a nasopharyngeal swab. For each of 3 presumptively pathogenic bacteria (*S. zooepidemicus, Actinobacillus/Pasteurella* spp and *S. pneumoniae*) and 2 non-pathogenic species (*Staphylococcus* spp. and *Acinetobacter* spp.), the proportions of tracheal washes that were positive for these bacteria were investigated at different levels of respiratory disease. As previously described (Table 5.1), disease categories were ordered according to inflammation score and presence/absence of clinical signs and data were analysed by chi square test for linear trend for increasing or decreasing proportions.

Results are summarised in Table 5.2 and showed that for all of the presumptively pathogenic species of bacteria there was a highly statistically significant linear trend for increasing proportions (and hence trend of increasing odds ratios) of positive isolation from tracheal washes as disease severity increased. However there was no evidence whatsoever of a similar trend for the transient, non-pathogenic bacterial species. As data were restricted to only those samplings when bacteria were recovered from the URT, these results indicate that it was highly unlikely that contamination of the trachea by bacteria from the URT was the only explanation for the presence of presumptively pathogenic bacteria in the trachea in diseased horses.

Several studies using different sampling techniques have apparently identified a proportion of horses that have low numbers or scanty growths of non-pathogenic bacteria in

the distal trachea or more caudal sites of the lung (Whitwell & Greet, 1984; Sweeney *et al.*, 1985a; Blunden & Mackintosh, 1991). Using transtracheal aspirates, one study found that 24% of 50 Thoroughbred racehorses and 64% of 36 pastured, non-racing animals in Philadelphia, USA had so called 'transient' non-pathogenic bacteria isolated from tracheal samples with *Staphylococcus* spp. and *Acinetobacter* spp. both isolated (Sweeney *et al.*, 1985a).

**Table 5.2: Proportions of tracheal washes (TWs) positive for bacterial species among only horses positive for the same organism on nasopharyngeal swab and ordered according to inflammation score and presence (cases) or absence (controls) of clinical signs**

| Case/Control | Inflammation score | TWs (%) positive for bacterial spp.* | Total TWs examined (100%) | Odds Ratio |
|---|---|---|---|---|
| *Streptococcus zooepidemicus* | | | | |
| Control | 0 | 21 (28%) | 74 | 1.00 (ref) |
| Case | 0 | 3 (30%) | 10 | 1.08 |
| Control | 1 | 28 (45%) | 62 | 2.08 |
| Case | 1 | 6 (60%) | 10 | 3.79 |
| Control | ≥2 | 30 (83%) | 36 | 12.6 |
| Case | ≥2 | 20 (71%) | 28 | 6.31 |
| $\chi^2$ for linear trend = 33.25 (P≤0.00001) | | | | |
| *Actinobacillus/Pasteurella* spp. | | | | |
| Control | 0 | 19 (22%) | 85 | 1.00 (ref) |
| Case | 0 | 4 (31%) | 13 | 1.54 |
| Control | 1 | 25 (32%) | 77 | 1.67 |
| Case | 1 | 6 (43%) | 14 | 2.61 |
| Control | ≥2 | 16 (80%) | 20 | 13.9 |
| Case | ≥2 | 13 (76%) | 17 | 11.3 |
| $\chi^2$ for linear trend = 30.28 (P≤0.00001) | | | | |
| *Streptococcus pneumoniae* | | | | |
| Control | 0 | 7 (24%) | 29 | 1.00 (ref) |
| Case | 0 | 1 (33%) | 3 | 1.57 |
| Control | 1 | 9 (32%) | 28 | 1.49 |
| Case | 1 | 2 (67%) | 3 | 6.29 |
| Control | ≥2 | 11 (69%) | 16 | 6.91 |
| Case | ≥2 | 7 (70%) | 10 | 7.33 |
| $\chi^2$ for linear trend = 11.85 (P=0.00058) | | | | |
| *Staphylococcus* spp. | | | | |
| Control | 0 | 110 (43%) | 253 | 1.00 (ref) |
| Case | 0 | 8 (28%) | 29 | 0.50 |
| Control | 1 | 88 (45%) | 197 | 1.05 |
| Case | 1 | 15 (45%) | 33 | 1.08 |
| Control | ≥2 | 32 (48%) | 67 | 1.19 |
| Case | ≥2 | 12 (29%) | 42 | 0.52 |
| $\chi^2$ for linear trend = 0.238 (P=0.63) | | | | |
| *Acinetobacter* spp. | | | | |
| Control | 0 | 8 (12%) | 67 | 1.00 (ref) |
| Case | 0 | 1 (14%) | 7 | 1.23 |
| Control | 1 | 7 (11%) | 63 | 0.92 |
| Case | 1 | 1 (11%) | 9 | 0.82 |
| Control | ≥2 | 10 (14%) | 74 | 1.15 |
| Case | ≥2 | 1 (5%) | 21 | 0.37 |
| $\chi^2$ for linear trend = 0.07 (P=0.80) | | | | |

Another study also considered that tracheal washes collected by endoscopy contained bacteria that were unlikely to be of pathogenic significance (Whitwell & Greet, 1984) and in a carefully conducted *post mortem* study, *Staphylococcus* spp. and *Acinetobacter* spp. were isolated from distal lung sites in 37% and 12% of horses

respectively (Blunden & Mackintosh, 1991). A difference was noted between different breeds of horses, with Thoroughbreds having a higher proportion positive for these bacteria than non-Thoroughbreds. This was thought to be attributable to differences in management practices between the breeds, but the important confounding effect of age was not considered. Together these studies suggest that the lower airways at least to the level of the carina and including the distal trachea, are not entirely sterile in all horses at all times and they do harbour transient, non-pathogenic bacteria periodically. It is likely that these bacteria are cleared naturally by normal mucociliary clearance mechanisms but they may be influenced by the presence of other pathogenic bacteria during the course of an infectious disease process. It is of note that in this study there was a strong inverse association between clinical respiratory disease and the presence of low numbers of some species of non-pathogenic bacteria in tracheal washes, including *Staphylococcus* spp. and *Acinetobacter* spp. The prevalence of non-sterile tracheal washes appears to vary according to sampling technique, with oropharyngeal contamination probably increasing the proportion of non-sterile samples when endoscopy is used. However, the methods used in quantification of bacteria in washes in this study were specifically designed to quantify all bacteria present in samples, including those that were likely to be acquired from contamination of the endoscope during its passage through the URT. Assuming that if cases were associated with increased URT bacterial numbers, then there would be correspondingly more contamination of tracheal washes in clinically affected horses than controls and the risk of being a case would increase with detection of transient bacterial species, which is contrary to the actual finding of this study.

Isolation of most bacterial species from the URT on nasopharyngeal swabs has not been shown to be associated with respiratory disease in horses, with the exceptions of *S. equi* and possibly *S. pneumoniae*. *Actinobacillus/Pasteurella* spp. and *S. zooepidemicus* are, therefore, generally considered to be normal URT commensal organisms and

considerable care is needed in interpreting their presence on URT swabs taken during clinical disease. In its overall findings this study failed to demonstrate a significant association between URT infection with bacteria and clinical respiratory disease. However, it is of note that in the final multivariable analysis using only 'healthy' controls, the presence of *Actinobacillus/Pasteurella* spp. on swabs as well as in tracheal washes was significantly associated with disease. Similarly to the situation in the trachea, there was also an inverse association shown with a non-pathogenic bacteria at the same time, this time with non-haemolytic *Streptococcus* spp.. We might suppose that the *Actinobacillus/Pasteurella* spp. isolated from the URT reflected increased numbers of the organism in the lower respiratory tract that were cleared by mucociliary mechanisms. However, further molecular epidemiological research is required to demonstrate that isolates of the same pathogenic bacteria recovered from both the upper and lower respiratory tract concurrently in horses suffering respiratory disease, are the same and therefore of common origin. It must be considered that the case definition used in this study also included signs of URT disease (Wood, 1999) and these URT bacteria may have been associated with this syndrome.

### 5.2.5 The effect of viral infections

This study failed to demonstrate a significant association between clinically apparent respiratory disease in young racehorses and infection with several species of viruses, as diagnosed by subsequent seroconversion. These infections included equine herpesvirus-1 (EHV-1), equine herpesvirus–4 (EHV-4), equine rhinovirus-1 (ERV-1) and equine adenovirus. Previous observational studies have found an apparent association between EHV-1 infection and URT disease as defined as nasal discharge (Powell *et al.*, 1978; Thomson, 1978; Morley, 1995; Burrell *et al.*, 1996). However, one study demonstrated that only around 10% of clinical cases had virus isolated and viruses were not found to be associated with subclinical outbreaks (Thomson, 1978). The results here agreed with a

recent Australian case control study that also failed to demonstrate any association between coughing and viral infections (Christley *et al.*, 1999b; 2001b).

The current study did identify that infection with equine influenza virus was significantly associated with clinical respiratory disease at a univariable level, although the prevalence of infection was extremely low among study animals (<1% prevalence overall). This very low prevalence among this population of horses was probably directly attributable to mandatory vaccination against the disease, which is required under the Jockey Club Rules of Racing for Thoroughbreds in the UK. However, influenza infection under the cover of vaccination does occur, particularly in young Thoroughbred racehorses, and produces both clinically apparent but mild as well as subclinical infections (Newton *et al.*, 1999a; 2000a). Serological diagnosis of influenza is possible in vaccinated horses (Newton *et al.*, 1999a; 2000a) and we assumed failure to seroconvert was a reliable indicator of non-infection and that the majority of horses in this study probably remained free of influenza infection. For the purposes of maximising available data and minimising the confounding effects of influenza infection in a very small number of horses in this well vaccinated population, all analyses in this study were conducted after exclusion of the only 2 matched sets in which seroconversion to influenza occurred.

## 5.3 Choice of study design

This case control study used a matched design to investigate the associations between clinically apparent respiratory disease and different infectious agents and other factors in young Thoroughbred racehorses in training in 3 training centres in the United Kingdom. As this study was designed to investigate associations between clinically presenting respiratory disease and a wide range of potential risk factors, particularly different infections, a conventional prospective cohort design would not have been appropriate. In such a cohort study, subjects of known exposure status to a limited number

of risk factors are followed over time to assess disease outcomes. In this case control study the exposure status of horses to many risk factors (i.e. the infections) changed over the study period.

Clinically apparent respiratory disease in young racehorses is recognised as a common and recurrent disease that does not confer solid immunity after a single episode (Burrell, 1985; Sweeney *et al.*, 1992). The restricted traditional epidemiological paradigm that the case control design was only useful for the study of risk factors for rare diseases is no longer accepted (Greenland & Thomas, 1982; Rodrigues & Kirkwood, 1990). In order for the case control design to be appropriate for the investigation of a prevalent disease such as respiratory disease in young racehorses, concurrent selection of controls was, therefore, necessary (Rodrigues & Kirkwood, 1990). Concurrent selection meant that subjects that had been cases at one time could also have been recruited as controls at a later stage (Rodrigues & Kirkwood, 1990). The longitudinal study of respiratory disease (Wood *et al.*, 1997b; Wood, 1999) allowed concurrent recruitment and investigation of horses that did (cases) and did not (controls) satisfy the case definition of clinically apparent respiratory disease. The design used was therefore similar to a nested case control study in which cases were recruited from a large cohort and compared to a restricted number of selected controls chosen from the same cohort (White *et al.*, 1998). As cases and controls were recruited from the same limited population (cohort) of horses in the participating training yards, this would help limit the chance that the selection of cases and controls was biased. The use of nested matched case control studies in the investigation of equine infectious respiratory disease has not been widespread to date although recent studies have used case control methods to investigate risk factors for pleuropneumonia (Austin *et al.*, 1995) and coughing (Christley *et al.*, 1999b; 2001a; 2001b) in horses. The study of risk factors for pleuropneumonia in American horses was somewhat different to the study reported here because it was retrospective in nature and was conducted among cases referred to a

teaching hospital (Austin *et al.*, 1995). However, the study of coughing among Thoroughbred racehorses in Sydney, Australia was much more similar in design to this study as it used prospective and concurrent recruitment of cases and controls with coughing cases being observed by the trainer or veterinary surgeon (Christley *et al.*, 1999b; 2001a; 2001b). Both this study and that conducted in Australia used an appropriate matched design with cases and controls being matched on trainer (and hence management factors) and time of sampling (i.e. concurrent selection).

## 5.4 Bias and confounding

There were several aspects of the design and conduct of this study that potentially introduced bias and confounding. The choice of case control study design, as discussed above, had considerable potential for the introduction of bias into this study. As the name indicates, case control studies require differentiation and comparison of cases and controls, therefore careful definition and classification of subjects into these groups was essential to minimise bias.

### 5.4.1 Definition and selection of cases

The definition of clinically apparent cases of respiratory disease in this study was based on any of 3 clinical parameters; coughing, nasal discharge or pyrexia, which in previous studies have been either strongly correlated with or used as a definition of respiratory disease (Wood & Mumford, 1992; Burrell *et al.*, 1994; Morley, 1995; Moore, 1996). However, this may be considered to be a non-specific definition, and although pyrexia may have a number of non-respiratory underlying causes (e.g. Mair & Lane, 1989), respiratory infection is generally considered to be the most likely cause of raised temperature in young horses. Case recruitment was not complete in this study because of the problems of ensuring compliance in busy training yards. This meant that missed cases

had the potential to be recruited as controls leading to bias and underestimation of the size of true effects. This was minimised by selecting participating yards where the trainer and veterinary surgeons were enlightened and concerned about respiratory disease and where there was a strong veterinary input.

There were differential case reporting rates between yards. There were probably many reasons for this including differences between trainers, veterinary surgeons, times of the year, the proximity of yards to the AHT and the convenience of deploying study investigators to sample clinically apparent cases. Matching on trainer and time of sampling helped control this. Within each yard there was possibly some bias in case selection due to trainers monitoring horses differently during critical periods of training such as in the build-up to races. This was considered a non-differential bias, however, as it was assumed that the trainer would generally apply the same investigation criteria to all horses irrespective of whether they were being routinely monitored.

It was apparent from results that there were more missing laboratory data from cases than controls because these animals were sampled at times other than routine monthly yard visits, usually by the yard's own veterinary surgeon who did not always submit a complete set of appropriate samples. Having cases but not controls examined by different veterinary surgeons may have contributed to a differential bias, although cases with missing data were more likely to be subsequently excluded in later analyses, thereby reducing the effect of this bias.

### 5.4.2 Definition and selection of controls

As only a sample of horses were selected for routine longitudinal monitoring of respiratory disease in each yard, there was the potential that the choice of these horses, which would be used as the source of controls, would introduce a bias. The sample of horses was chosen by the trainer to be broadly representative of the age structure, housing

and general management of the yard. Although their initial selection was not necessarily random it was probable that this effect would diminish over time as recurrent disease occurred. As cases were recruited from the entire yard and controls only from the longitudinal sample of horses there was potential for biased selection of cases and controls if unmeasured factors within yards were influencing the occurrence of disease differently in each group. As the longitudinal sample of horses was generally representative of the whole yard, the within-yard effects, which would not be controlled by matching for trainer, were not likely to be significant.

The allocation into subgroups of controls, classified as 'healthy' or 'subclinical', was done after the random selection, subject to the requirements for matching, of the entire control group, which in turn was done irrespective of respiratory health status. Therefore, there was no additional bias introduced by the selection of these control subgroups.

There was an absence of strict exclusion criteria for controls in this case control study because the investigation was essentially undertaken after all the examinations and sampling had been conducted. Therefore horses were not classified as cases or controls prospectively acording to strictly applied criteria. Case identification was, therefore, reliant on later checking records of clinical signs kept at the time of sampling to identify animals that were subsequently classified as cases. In the absence of clinical signs being recorded the remainder of horses were automatically classified as controls. This might inevitably have lead to some misclassification of both cases and controls in this study.

## 5.5 Conclusions

This matched case control study demonstrated that clinically apparent respiratory disease in young Thoroughbred racehorses was statistically associated with several infectious and non-infectious risk factors. Younger horses and those that had entered training within the last 3 months and those that had *Actinobacillus/Pasteurella* spp. or

*Mycoplasma felis* isolated from the trachea were at increased risk of suffering clinical respiratory disease. *Streptococcus zooepidemicus* in the trachea was significantly associated with both subclinical IAD in controls and clinical respiratory disease in cases when compared with controls with no endoscopic or cytological evidence of IAD. There was evidence for an inverse association between clinical disease and the presence of small numbers of transient, probably non-pathogenic bacteria in the trachea, although the true significance of this is not clear. There was no significant association found between viral infections and clinical disease other than for equine influenza, which was an extremely rare infection in this well vaccinated and managed population. After controlling for other factors, no significant association was identified between previous or recent racing and clinical disease. The only significant upper respiratory tract bacterial infection associated with respiratory disease was *Actinobacillus/Pasteurella* spp., which was identified only when clinical cases were compared with horses with no endoscopic or cytological evidence of IAD. This study demonstrates that the horse's age, its time since entry into training and bacterial and mycoplasma infections of the trachea are important factors in clinically presenting respiratory disease in flat trained racehorses. Several aspects of these factors require further investigation to better understand their role in the epidemiology of equine inflammatory respiratory disease.

# SECTION 3

# A STUDY OF NATURALLY OCCURRING RESPIRATORY DISEASE IN WELSH MOUNTAIN PONY FOALS

# CHAPTER 6

# INTRODUCTION

## 6.1 Background

During many years of the AHT acquiring recently weaned Welsh Mountain pony foals for use in various types of studies, it was clear that these young animals consistently suffered a significant burden of natural respiratory disease that was not associated with infection by known equine viruses (AHT unpublished observations). The disease, which occurred soon after ponies were mixed and transported from Wales to Suffolk, was characterised by both obvious clinical signs and lower respiratory tract inflammation. The disease was invariably associated with bacterial infections, particularly with *Streptococcus zooepidemicus, Streptococcus pneumoniae* and *Actinobacillus/Pasteurella* spp. and in most cases apparently persisted for several weeks.

In order to study this respiratory disease phenomenon in young Welsh Mountain ponies in more detail, a study was conducted by Dr. Neil Chanter in collaboration with the research and development arm of a commercial pharmaceutical company (Hoechst Roussel Vet).

## 6.2 Aims of the study

The overall aim of the study was to test the efficacy of a multiple strain experimental bacterial vaccine against naturally occurring respiratory disease in recently weaned pony foals following mixing and transportation to the AHT. Although Dr. Chanter had conducted simple descriptions of study data in terms of the proportion of ponies affected by clinical signs and infections over the study period, more detailed analyses of the data were required and these are presented here.

After the study was unblinded it seemed to Dr Chanter that some ponies encountered less disease whereas others were clearly more susceptible, irrespective of vaccination status. Stimulated by Dr Chanter's impressions of a difference in susceptibility to respiratory disease among these ponies, discussions were conducted with immunogeneticists at the Eighth Equine Infectious Diseases Conference in Dubai in March 1998 regarding equine transferrin as a source of iron for growth of *Actinobacillus equuli*. This led to the suggestion that variations in transferrin haplotypes may influence the ability of *A. equuli* to acquire iron and hence to differences in the susceptibilities of individual ponies to respiratory disease. The transferrin haplotypes of ponies were consequently determined and these, with protease inhibitor haplotypes as a 'control' variable (i.e. as they are also used during equine blood typing but have no obvious biological basis for inclusion), were examined as pony-level risk factors in analyses. Other variables, including autoregressive outcomes, tracheal and nasopharyngeal infections, sex and vaccination status were also evaluated.

# CHAPTER 7

# MATERIALS AND METHODS

## 7.1 Study design

The study was a randomised, double blinded, placebo controlled trial of an alum adjuvanted vaccine containing 2 killed strains of *S. zooepidemicus* and 2 killed strains of *A. equuli*. The sterile placebo was identical to the vaccine in all aspects other than it did not contain bacteria. Study ponies, selected from a single location in Wales, each received 2 doses of intramuscularly administered vaccine or placebo at 4-week intervals whilst at an interim holding site in Shropshire. Several days after the second dose, the study ponies were mixed with 5 other ponies from 5 distinct sites across Wales and transported together in a single batch by road to the study site near Newmarket, Suffolk. All ponies were then monitored by twice weekly detailed clinical examination and sampled once a week by nasopharyngeal swab, endoscopy and tracheal wash sampling. Blood samples for viral serology were taken at monthly intervals. Examinations and sampling continued for 10 consecutive weeks after ponies were moved to the study site and a final examination and sampling was conducted 16 weeks later (i.e. 26 weeks after mixing and transportation). I conducted all vaccinations and these were blinded from Dr. Neil Chanter, the principal investigator, who conducted all clinical examinations and samplings during the study. The study was conducted under appropriate Home Office licences.

### 7.1.1 Welsh Mountain ponies

A group of 24 recently weaned, 4 to 6 month old, Welsh Mountain pony foals, comprising 10 males and 14 females, were recruited for the study. Ponies were randomly allocated to 2 equally sized groups (n=12) to receive either vaccine or placebo and the

principal investigator remained blinded to the randomisation code throughout the duration of the study.

All 24 ponies were acquired from a geographically very small area i.e. they ran together on the same Welsh hill, which would theoretically reduce confounding from immunity from previous infections. Selecting vaccinates and controls from the same small geographic area would mean they were likely to have had less exposure to multiple pathogens and that any measurable difference in disease between the groups would be attributable to vaccination rather than immunity acquired by previous infection. Horses from a geographically small area would theoretically reduce the number of subtypes of bacterial strains that ponies would have been exposed to prior to vaccination and increase the chances of meeting new subtypes when mixed and transported with other ponies from other geographically diverse areas. Mixing of vaccinates/controls with a third group of ponies from diverse locations was intended to increase the likelihood that they would encounter markedly different infections following vaccination. Any subsequent measurable differences in disease severity between the vaccine and placebo groups might then be attributable to immunity from vaccination. Use of ponies from diverse locations would have increased the likelihood of them having markedly different previous infections and any measurable differences in disease severity between the vaccine and placebo groups might be attributable to differences in previously acquired immunity rather than to immunity from vaccination.

A third group of 5 Welsh Mountain ponies from 5 separate and geographically diverse locations within Wales was mixed with the vaccine and placebo groups after vaccination but several days before all 29 ponies were transported together by road to Newmarket. Detailed examination and sampling of all ponies over the next 10 weeks would test whether the vaccinated ponies had increased immunity to these novel infections compared with the placebo group.

### 7.1.2 Twice weekly clinical examinations

Clinical examinations followed a standard protocol which included measurement of rectal temperature, nasal and ocular discharges, coughing, abnormal breathing pattern and submandibular lymphadenopathy. Individual clinical parameters (signs) were assigned a score according to severity and these were recorded on an individual record sheet for each pony at each examination (Table 7.1).

**Table 7.1: Summary of scoring recorded at the time of clinical assessment for individual clinical parameters**

| Clinical parameter | Scoring allocation | Possible maximum |
|---|---|---|
| Nasal discharge | Normal=0, serous=1, unilateral=1, mucopurulent=2, bilateral=2 | 4 |
| Ocular discharge | Normal=0, serous=1, unilateral=1, mucopurulent=2, bilateral=2 | 4 |
| Coughing | Absent during examination=0, present during examination=2 | 2 |
| Breathing pattern | Normal=0, dyspnoea/abdominal breathing=2 | 2 |
| Submandibular LNs | Normal=0, swollen=1 | 1 |

### 7.1.3 Weekly sampling

On a single occasion during each week of the study all ponies were sampled and visual examination of the trachea was made using an endoscope. Similar samples were taken as those described for the longitudinal study of respiratory disease in Thoroughbred racehorses.

Blood samples were taken prior to vaccination and at 2, 6 and 10 weeks after transportation for serological testing for antibodies against equine respiratory viruses. Nasopharyngeal swabs were taken for bacteriological testing including PCR typing of isolates of *S. zooepidemicus*. Swabs were immediately placed in transport medium and were kept cool on ice blocks in a polystyrene box for transportation over the short distance to the

laboratory. A 1.0m flexible endoscope was used to evaluate the amount of mucus visible in the trachea and this was recorded on a dedicated recording sheet as either absent (score = 0), slight (score = 1), moderate (score = 2) or severe (score = 3). A tracheal wash sample using PBS was collected and transported to the laboratory on ice blocks. Thoroughly mixed aliquots were used for nucleated and red blood cell counts (EDTA sample), cytological examination (formalin fixed sample) and quantitative bacteriology and *S. zooepidemicus* typing PCR. The endoscope was thoroughly cleaned and disinfected between each pony.

## 7.2 Data

The data consisted of 11 weekly (repeated) observations (i.e. week of observation after mixing with the non-vaccinated group) for all 29 ponies, giving a total of 319 observations. The ponies were categorised according to whether they received vaccine, placebo or were introduced later.

### 7.2.1 Pony level variables

There were several variables which resided at the pony level and which, therefore, did not alter over the weeks of observation. These variables included sex, vaccine group and each pony's transferrin and protease inhibitor haplotype derived from typing of blood samples from each pony .

Sex was recorded as a binary variable with male (1) and female (0) categories and vaccine group as a categorical variable with vaccine (1), control (2) and introduced later (3) categories. Variables corresponding to each of the transferrin (D, F2, H1, H2, O & R) and protease inhibitor (I, L, L2, R & S) haplotypes were recorded as binary variables, i.e. corresponding to the presence (1) or absence (0) of that particular haplotype, independent

of the presence or absence of any other haplotype in that individual. Table 7.2 summarises the distribution of these pony level variables among each sex and in total.

**Table 7.2: Overall and sex-specific distribution of pony level variables and frequency of specific transferrin and protease inhibitor phenotypes**

| Pony level variable | Female (n) | Male (n) | Total (n) |
|---|---|---|---|
| **Total** | 15 | 14 | 29 |
| **Vaccine group** | | | |
| Vaccine (V) | 6 | 6 | 12 |
| Placebo (P) | 8 | 4 | 12 |
| Introduced later (I/L) | 1 | 4 | 5 |
| **Transferrin haplotype positive** | | | |
| D | 9 | 9 | 18 |
| F2 | 9 | 5 | 14 |
| H1 | 2 | 1 | 3 |
| H2 | 1 | 3 | 4 |
| O | 5 | 4 | 9 |
| R | 3 | 1 | 4 |
| **By vaccine group →** | **V** | **P** | **I/L** |
| D | 9 | 8 | 1 |
| F2 | 5 | 4 | 5 |
| H1 | 1 | 2 | 0 |
| H2 | 2 | 2 | 0 |
| O | 3 | 6 | 0 |
| R | 2 | 2 | 0 |
| **Protease inhibitor haplotype positive** | | | |
| I | 8 | 5 | 13 |
| L | 13 | 13 | 26 |
| L2 | 1 | 0 | 1 |
| R | 0 | 1 | 1 |
| S | 1 | 4 | 5 |

| Frequency of specific phenotypes | | | | |
|---|---|---|---|---|
| Transferrin type | | N | PI type | n |
| D | O | 5 | L L | 10 |
| D | F2 | 4 | L I | 10 |
| F2 | F2 | 4 | L S | 4 |
| D | R | 3 | I I | 2 |
| D | H2 | 3 | L L2 | 1 |
| F2 | O | 3 | L R | 1 |
| D | D | 2 | I S | 1 |
| F2 | H1 | 2 | | |
| D | H1 | 1 | | |
| H2 | O | 1 | | |
| F2 | R | 1 | | |
| **Total** | | **29** | | **29** |

PI = protease inhibitor

### 7.2.2 Observation level variables

There were a series of both outcome and explanatory variables that were specific to each time of observation for each pony.

### *7.2.2.1 Outcome variables*

The outcome variables of interest corresponded to individual and aggregated clinical scores allocated to severity of particular signs of disease. Individual clinical parameter scores were recorded on dedicated scoring sheets at the time of clinical evaluation (Table 7.1) for nasal and ocular discharges, coughing, abnormal breathing/dyspnoea and submandibular lymph node (SMLN) enlargement.

Aggregated clinical and airway inflammation scores

Two aggregated clinical scores were used as overall summary measures of severity of clinical respiratory disease. Clinical score was the term used for the sum of all 5 individual clinical parameters whereas CDNS score was the sum of coughing (C), abnormal breathing/dyspnoea (D), nasal discharge (N) and SMLN enlargement (S) but excluded ocular discharge. The alternative score of CDNS was also used because ocular discharge may not have reflected respiratory disease *per se*, but possibly some environmental influence such as hay dust encountered whilst ponies fed from hay racks. Comparison of analyses including and excluding ocular discharge in aggregated scores might allow this possibility to be evaluated.

Although clinical observations were conducted twice weekly, respiratory sampling was only conducted once a week and this was always done at the time of the first clinical assessment. Therefore in order that numbers of clinical and sampling observations corresponded, a single weekly average (mean) was calculated for clinical parameters. For the purposes of this study the weekly average was considered the most appropriate measure of the weekly clinical severity of respiratory disease, with each of the 2 examinations being weighted equally.

Table 7.3 summarises the scoring system used for analysis purposes for individual and aggregated clinical parameters. In order that scores for nasal and ocular discharges

were not overly weighted in the aggregated clinical scores (each originally counting for a score of up to 4 compared to 2 or 1 for other clinical signs Table 7.1), the scoring allocations for these 2 clinical parameters were halved. This, therefore, gave a possible maximum score of 2 for nasal and ocular discharges that were mucopurulent and bilateral, with the possibility of lower scores for serous and/or unilateral discharges. The clinical signs of coughing and abnormal breathing/dyspnoea were considered to be clinically equivalent in their contribution to respiratory signs to bilateral, mucopurulent discharges and were each scored 2 when present. Enlargement of the submandibular lymph nodes was considered less clinically significant than coughing, abnormal breathing/dyspnoea and bilateral, mucopurulent discharges and was consequently attributed a score of one when present.

Overall individual observation scores (n=319) were taken as the average of 2 weekly examination scores and aggregated weekly clinical scores were equivalent to the sum of the relevant average individual parameter scores. This averaging over the 2 clinical examinations increased the precision of aggregated scores because of the possibility of fractional scores. For example, a pony that had clinical scores of 5 and 6 during 2 examinations in a particular week, would have an averaged weekly clinical score of (5+6)/2 = 5.5 and this would be out of a possible maximum of 9 (i.e. (9+9)/2).

**Table 7.3: Summary of scoring used for analyses for individual and aggregated clinical parameters**

| Clinical parameter | Scoring allocation per clinical examination | Maximum possible score |
|---|---|---|
| **Individual parameters** | | |
| 1. Nasal discharge* | normal=0, serous=0.5, unilateral=0.5, mucopurulent=1, bilateral=1 | 2 |
| 2. Ocular discharge* | normal=0, serous=0.5, unilateral=0.5, mucopurulent=1, bilateral=1 | 2 |
| 3. Coughing | absent during examination=0, present during examination=2 | 2 |
| 4. Breathing | normal=0, dyspnoea/abdominal breathing=2 | 2 |
| 5. SMLN | normal=0, enlarged/swollen=1 | 1 |
| **Aggregated scores** | | |
| Clinical score | sum of scores for all 5 clinical parameters | 9 |
| CDNS score | sum of scores for cough, breathing, nasal discharge & SMLNs only | 7 |

*modified nasal and ocular discharge scores from those recorded, SMLN = submandibular lymph nodes

In addition to the outcome measures of clinical and CDNS scores, another outcome measuring airway inflammation was derived from 3 component observations. Airway inflammation score was derived from the sum of scores for i) mucus visible in the trachea on endoscopy, ii) the density of cells on a smear of tracheal wash examined cytologically and iii) the relative proportion of all cells that were neutrophils on the same smear. Table 7.4 summarises the allocation of scores to different categories of the component observation scores, which have been in common usage by clinicians and pathologists at the AHT over many years and have proved reliable semi-quantitative measures. In analyses airway inflammation score was treated as an ordinal variable.

**Table 7.4: Summary of component observation scores for airway inflammation score**

| Tracheal mucus score (Mucus) | Tracheal wash smear cell density (SCD) | Neutrophil proportion in tracheal wash (Neutrophil) |
|---|---|---|
| 0 no mucus visible | 0 low density | 0 no cells observed |
| 1 slight mucus visible | 0 medium-low density | 0 few cells seen |
| 2 moderate mucus visible | 1 medium density | 0 small numbers and diffuse |
| 3 severe mucus visible | 2 medium-high density | 1 small numbers but aggregated |
| | 3 high density | 2 moderate proportion of cells |
| | | 3 predominant cell type seen |
| **Airway inflammation score = Mucus + SCD + Neutrophil** | | |

Individual clinical signs

For each individual clinical parameter, a binary outcome was generated corresponding to whether the clinical sign was considered present during the week of observation (1) or not (0). For coughing, abnormal breathing and submandibular lymph nodes, ponies possessing the sign at either of the clinical examinations during the week were classified as having that sign and this corresponded to an average parameter score >0. However, a more discretionary cut-off was used for nasal and ocular discharges as these

parameters were scored for their nature (absent, serous or mucoid) and extent (absent, unilateral and bilateral) rather than simply just presence or absence.

Table 7.5 summarises the derivation of binary outcomes for both nasal and ocular discharges from all of the possible combinations of discharge scores from 2 weekly examinations (weekly average scores).

**Table 7.5: Summary of the derivation of binary outcome scores for nasal and ocular discharges from combinations of discharge scores from 2 weekly examinations**

| | Examination 1 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | | Unilateral serous | | Bilateral serous | | Unilateral mucoid | | Bilateral mucoid | |
| **Examination 2** | WAS | Binary | WAS | Binary | WAS | Binary | WAS | Binary | WAS | Binary |
| Normal | 0.0 | 0 | 0.5 | 0 | 0.75 | 1 | 0.75 | 1 | 1.0 | 1 |
| Unilateral serous | 0.5 | 0 | 1.0 | 0 | 1.25 | 1 | 1.25 | 1 | 1.5 | 1 |
| Bilateral serous | 0.75 | 1 | 1.25 | 1 | 1.5 | 1 | 1.5 | 1 | 1.75 | 1 |
| Unilateral mucoid | 0.75 | 1 | 1.25 | 1 | 1.5 | 1 | 1.5 | 1 | 1.75 | 1 |
| Bilateral mucoid | 1.0 | 1 | 1.5 | 1 | 1.75 | 1 | 1.75 | 1 | 2.0 | 1 |

WAS = Weekly Average Score, Binary = Binary Outcome Score

Animals that were normal on both examinations (weekly average score=0) or had a unilateral, serous discharge on one (weekly average score=0.5) or both (weekly average score=1.0) occasions were all considered not to have had a nasal or ocular discharge during that week. Ponies with all other combinations of signs were classified as having had nasal or ocular discharge.

### *7.2.2.2 Explanatory variables*

The explanatory variables of interest at the observation level corresponded to results of microbiological examination of tracheal wash and nasopharyngeal swabs taken at the time of observation/sampling.

Tracheal washes had individual species of bacteria enumerated and expressed as colony forming units (cfu) per ml of sample and total bacterial count was the sum of the total counts of each species, also expressed as cfu/ml. Quantitative data were available at each sampling for total bacteria, *S. zooepidemicus, A. equuli, Pasteurella* spp., *Bordetella bronchiseptica* and non-haemolytic *Streptococcus* spp. For analytical purposes, quantitative bacteriology results (cfu/ml) for all samples were transformed by taking the $\log_{10}$ of their value plus one.

In addition, autoregressive variables, corresponding to the clinical outcome of interest (i.e. aggregated scores or individual clinical binary outcomes) at previous sampling points (1, 2 and 3 weeks previously), were generated. Inclusion of these variables, however, did limit the amount of available data for analyses. For example, autoregressive variables for the outcome the previous week were only available from the second sampling onwards, for outcomes 2 weeks previously from the third week onwards and for outcomes 3 weeks previously from the 4th week onwards. In addition, autoregressive variables for the sampling at week 26 were not deemed relevant, as this would refer to samplings at least 16 weeks earlier.

For analytical purposes bacterial isolate data for nasopharyngeal swab samples were expressed in a binary manner, corresponding to whether individual species of bacteria were isolated (1) or not (0) from swabs. Bacteria isolated from nasopharyngeal swabs included *S. zooepidemicus*, *Pasteurella* spp., *Bordetella bronchiseptica*, non-haemolytic *Streptococcus* spp. and *Staphylococcus* spp.. However, *A. equuli* was not isolated from nasopharyngeal swabs during the course of the study.

### 7.2.3 Pony level cumulative summary measures for repeated observations

In order to conduct a preliminary examination of data at the pony level (prior to analysis of individual observations, which considered repeated observations from specific ponies), cumulative summary measures of outcome and explanatory variables were derived from the data as explained in Table 7.6. Cumulative summary measures for each individual clinical outcome were the sum of scores for each clinical sign and were treated as continuous data. Similarly, cumulative aggregated scores were also generated and were also treated as continuous data. Various cumulative summary measures for infectious explanatory variables were derived for different infections and also took the form of continuous data. These included the total, maximum and geometric mean $\log_{10}$ count for specific bacterial species in tracheal wash samples and the $\log_{10}$ count at week 26 of the study. In addition, the total number of weeks that each horse had counts of specific bacteria in washes at greater than $10^3$, $10^4$ and $10^5$ cfu/ml were calculated.

It was considered valid to include clinical and infectious examination results at week 26 in the cumulative summary measures as all animals had been examined at this time and analyses of these data would investigate differences between ponies (n=29) and not observations (n=319) within the cumulative data.

**Table 7.6: Details of pony level cumulative summary measures for clinical and infectious variables**

| Pony level summary measure | Derivation |
|---|---|
| **Clinical outcome variables** | |
| Nasal discharge (ndavg) | Sum of 11 average weekly scores |
| Ocular discharge (odavg) | Sum of 11 average weekly scores |
| Coughing (coughavg) | Sum of 11 average weekly scores |
| Breathing (dyspavg) | Sum of 11 average weekly scores |
| SMLN (smlnavg) | Sum of 11 average weekly scores |
| Clinical score (clinsc) | Sum of all scores for all weeks |
| CDNS score (cdns) | Sum of cough, breathing, nasal discharge & SMLN scores for all weeks |
| **Infectious explanatory variables** | **(each for *S. zooepidemicus, A. equuli, Pasteurella* spp., *B. Bronchiseptica* and non-haemolytic *Streptococcus* spp.)** |
| Total $\log_{10}$ cfu/ml | $\text{Log}_{10}$ of sum of 11 weekly arithmetic cfu/ml counts |
| Maximum $\log_{10}$ cfu/ml | Maximum $\log_{10}$ count encountered through the study period |
| Geometric mean $\log_{10}$ cfu/ml | Mean of $\log_{10}$ weekly counts |
| Total weeks with $\geq 10^3$ cfu/ml | Sum of number of weeks with equal to or greater than $10^3$ cfu/ml |
| Total weeks with $\geq 10^4$ cfu/ml | Sum of number of weeks with equal to or greater than $10^4$ cfu/ml |
| Total weeks with $\geq 10^5$ cfu/ml | Sum of number of weeks with equal to or greater than $10^5$ cfu/ml |
| Week 26 $\log_{10}$ cfu/ml | $\text{Log}_{10}$ cfu/ml in sample taken at week 26 |

## 7.3 Statistical analyses

### 7.3.1 Pony level analyses

Data were examined to see whether there were any statistically significant differences between measures of clinical outcome (continuous clinical score data) among different categories of individual explanatory variables.

The distribution of data for pony level clinical scores (Figure 7.1) and CDNS scores (Figure 7.2) were examined by normal quantile-quantile (QQ) plots and were considered satisfactorily normally distributed and did not require transformation.

**Figure 7.1: Normal quantile-quantile plot for pony level clinical scores**

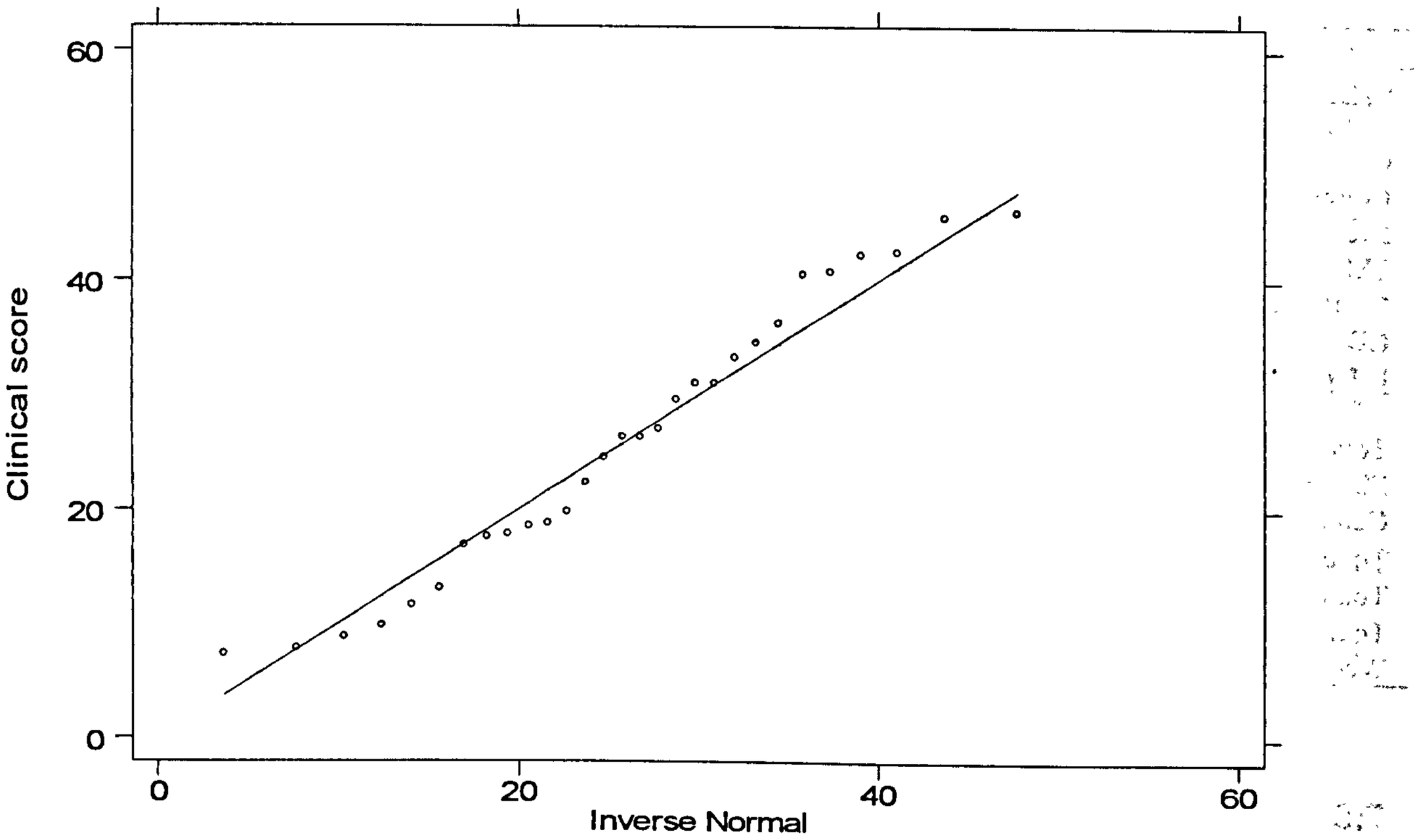
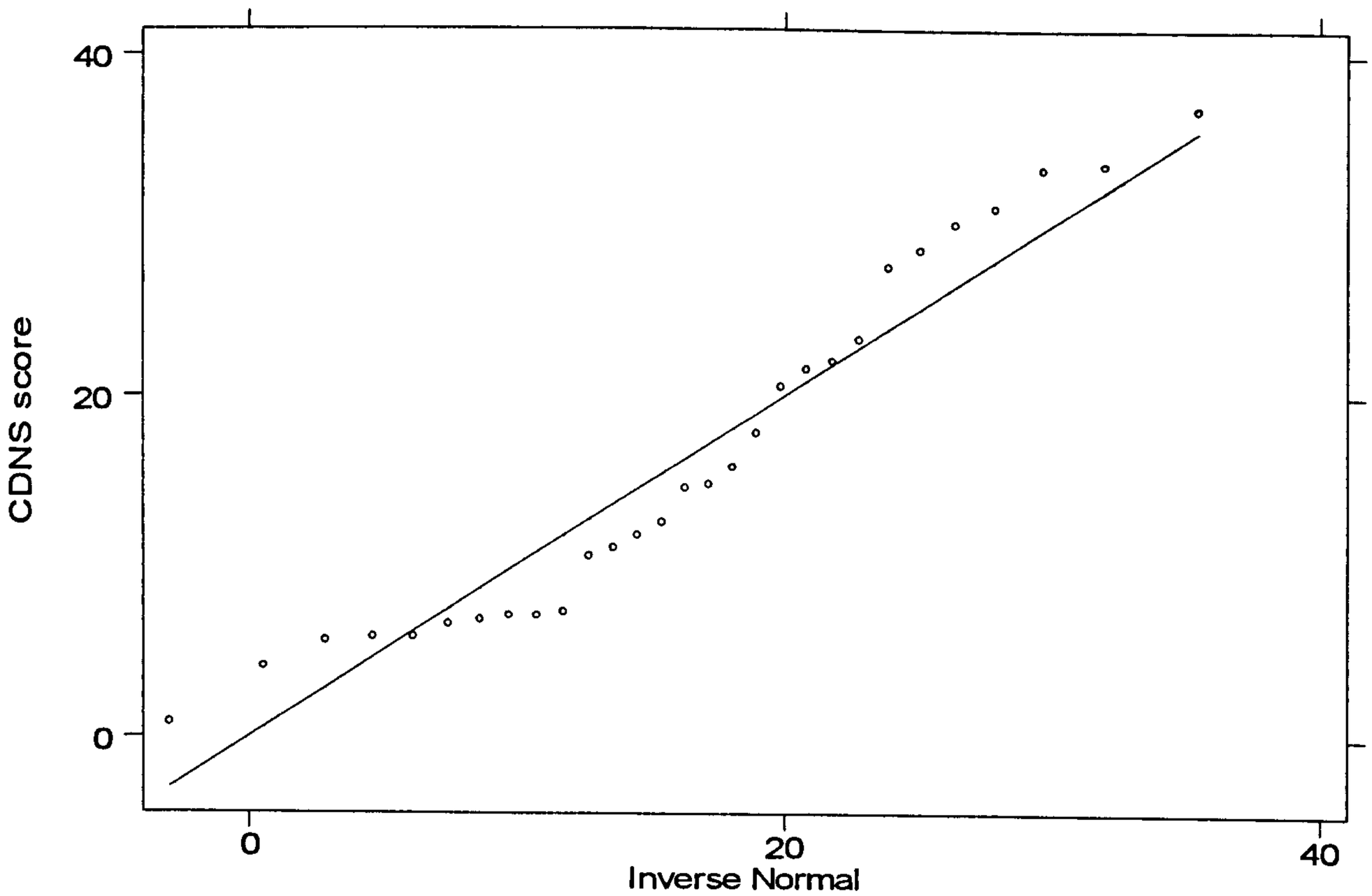



**Figure 7.2: Normal quantile-quantile plot for pony level CDNS scores**



The more conservative non-parametric ranking methods of the Wilcoxon rank sum test (for binary variables such as sex and possession of each transferrin and protease inhibitor haplotype) and the Kruskal-Wallis test (for categorical variables such as vaccine group) were used. Statistical significance was arbitrarily set at the 5% level. Where significant differences were identified, the direction of the difference in cumulative clinical outcome score in relation to the categories of the explanatory variable was examined.

Continuous summary measures of infectious explanatory variables were examined for their association with continuous cumulative clinical outcomes using simple linear regression. Linear regression was used to describe data by the following equation:

$$\text{outcome } y_{pony} = \alpha + \beta_{1pony}x_1$$

where $\alpha$ = intercept estimate

$\beta_{1pony}$ = slope estimate for variable $x_1$

Where results indicated a significant association between multiple variables, stratified analyses were then performed in order to examine the nature of the relationship between variables according to the different levels of another parameter. For example, to investigate whether the variation in scores between sexes was explained by the effect of a particular transferrin group or *vice versa*, a stratified analysis was conducted. In this stratified approach, the effect of sex for each category of a particular transferrin haplotype (present or absent) and the effect of transferrin haplotype for each sex were estimated.

Having examined the relationship between pony-level outcome and explanatory variables, the relationship between several of the explanatory variables was further investigated. In particular, data were examined for any significant differences between cumulative summary measures of infectious variables and sex, vaccine group and possession of transferrin and protease inhibitor haplotypes. Again the more conservative non-

parametric techniques of the Wilcoxon rank sum test and the Kruskal-Wallis test were used. All pony level analyses were conducted using Stata5.0 computer software (Stata Corporation).

There were no missing data for clinical outcomes in this study as all ponies were clinically examined on all occasions and hence there were no missing values for cumulative outcome variables. However, there were 5 occasions when tracheal wash samples could not be taken and these missing infectious data were ignored in generating cumulative explanatory variables for all parameters except the week 26 bacterial counts i.e. variables were generated for the number of weeks for which there were data available. This was considered preferable to excluding all pony-level data for ponies that had missing tracheal washes as this would considerably reduce the effective sample size. It was also considered that the missing samples occurred randomly and as such were unlikely to introduce a significant bias by underestimation of variable values. There was no tracheal wash taken from pony 21 on week 26 and so the week 26 bacterial counts for this pony were set as missing values and all analyses using these variables were based on 28 rather than 29 observations (ponies).

### 7.3.2 Observation level analyses

#### *7.3.2.1 Aggregated clinical sign and airway inflammation scores*

Three aggregated outcome measures of respiratory disease were generated for each observation (n=319), with an observation corresponding to a week of the study (n=11) for each pony (n=29). Clinical score was the sum of each of the weekly average scores for the 5 individual clinical signs of nasal and ocular discharges, coughing, abnormal breathing/dyspnoea and submandibular lymph node enlargement. CDNS score was the sum of the same individual sign scores but excluding the score for ocular discharge. Airway inflammation score was the sum of scores for endoscopically visible tracheal mucopus,

cytological smear cell density and neutrophil predominance in tracheal wash smears. Aggregated clinical sign and airway inflammation scores were treated as continuous variables.

The distribution of data for clinical scores (Figure 7.3), CDNS scores (Figure 7.4) and inflammation scores (Figure 7.5) were examined by normal quantile-quantile (QQ) plots. Although these data were not perfectly normally distributed due to an excess of zero values at the lower end of the distributions, the use of logarithmic transformations, usually applied to positively skewed data such as these, did not make sufficient differences to the distributions to merit using transformed data.

**Figure 7.3: Normal quantile-quantile plot for observation level clinical scores**

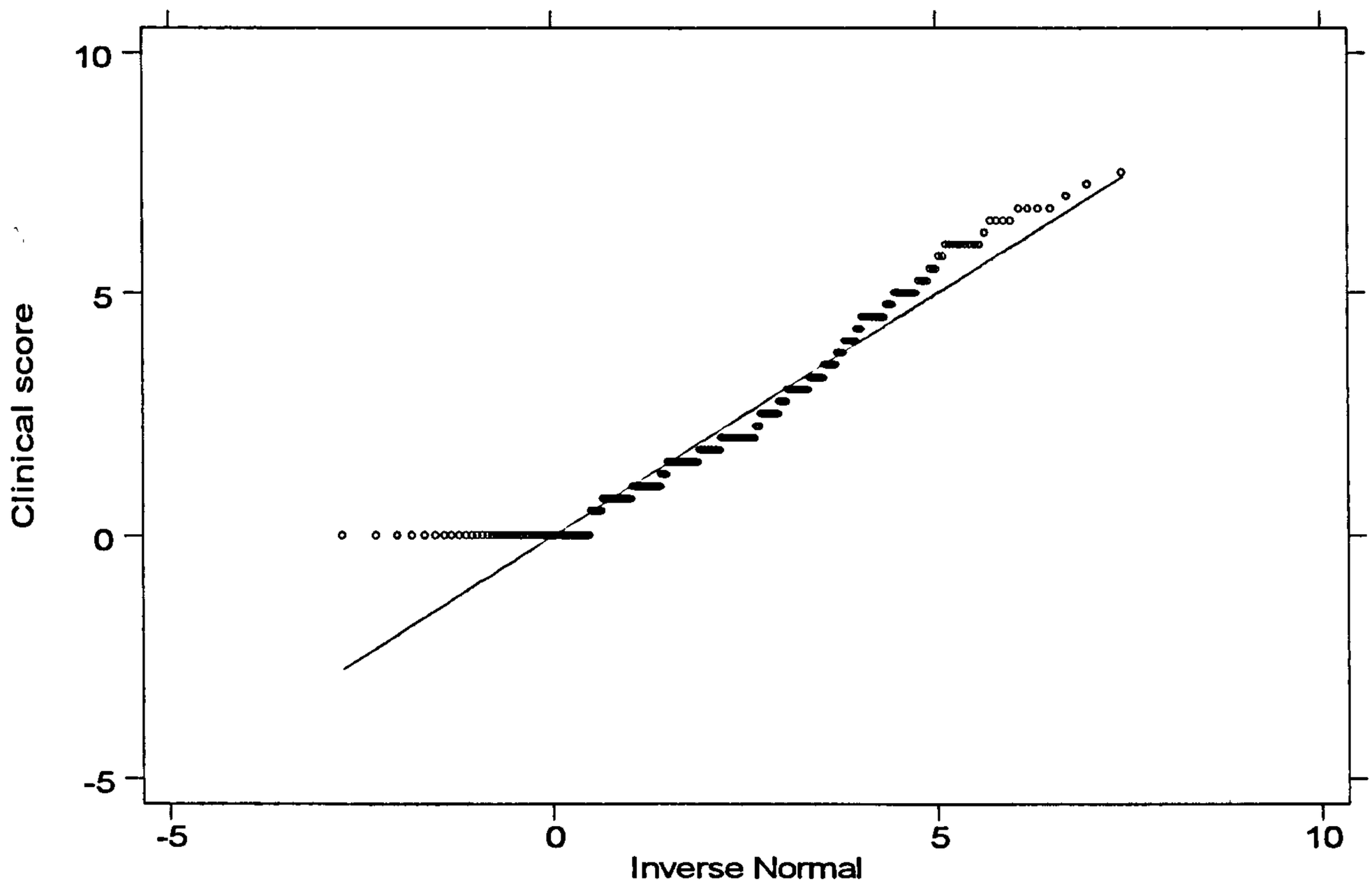


**Figure 7.4: Normal quantile-quantile plot for observation level CDNS scores**

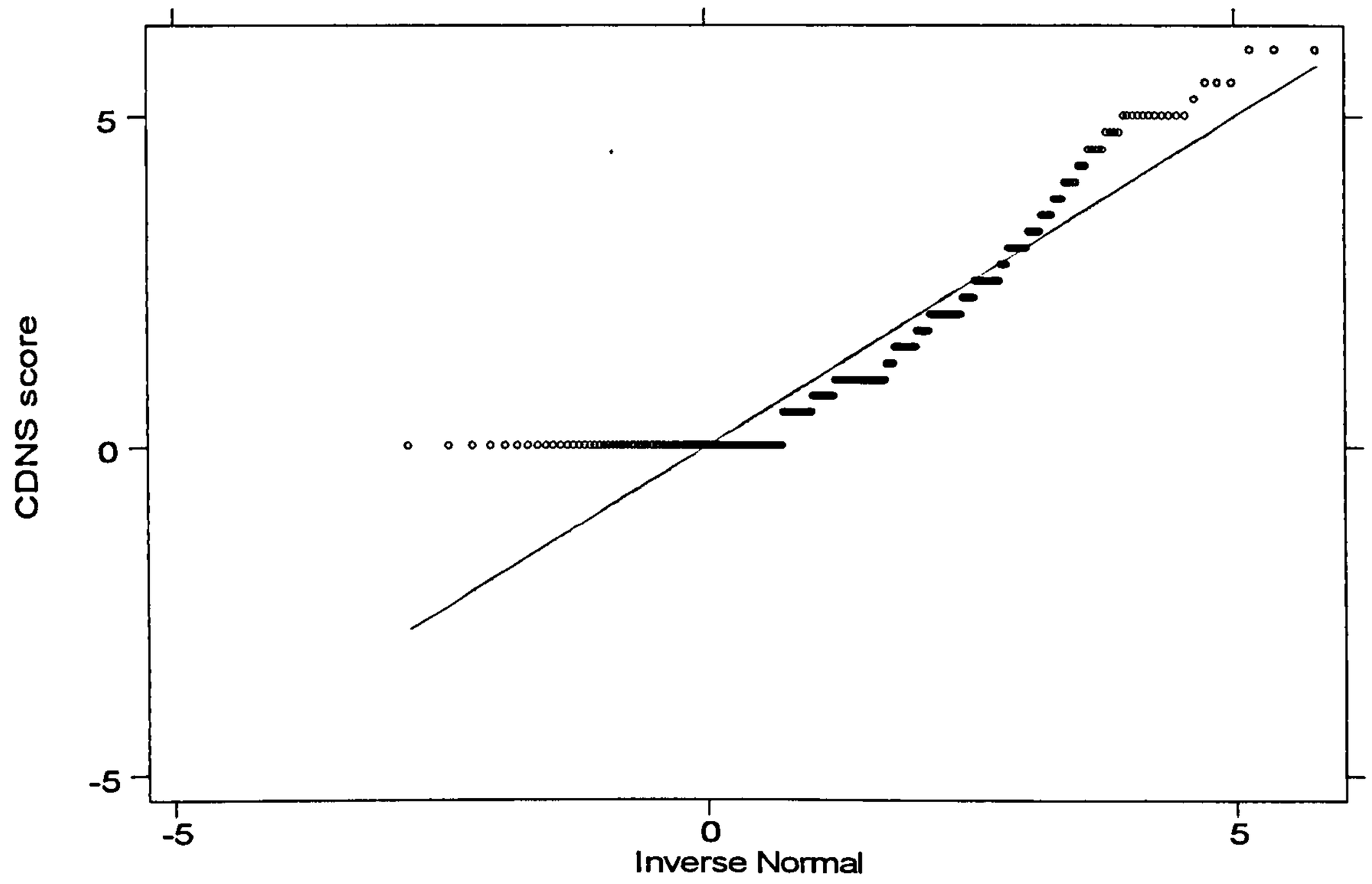
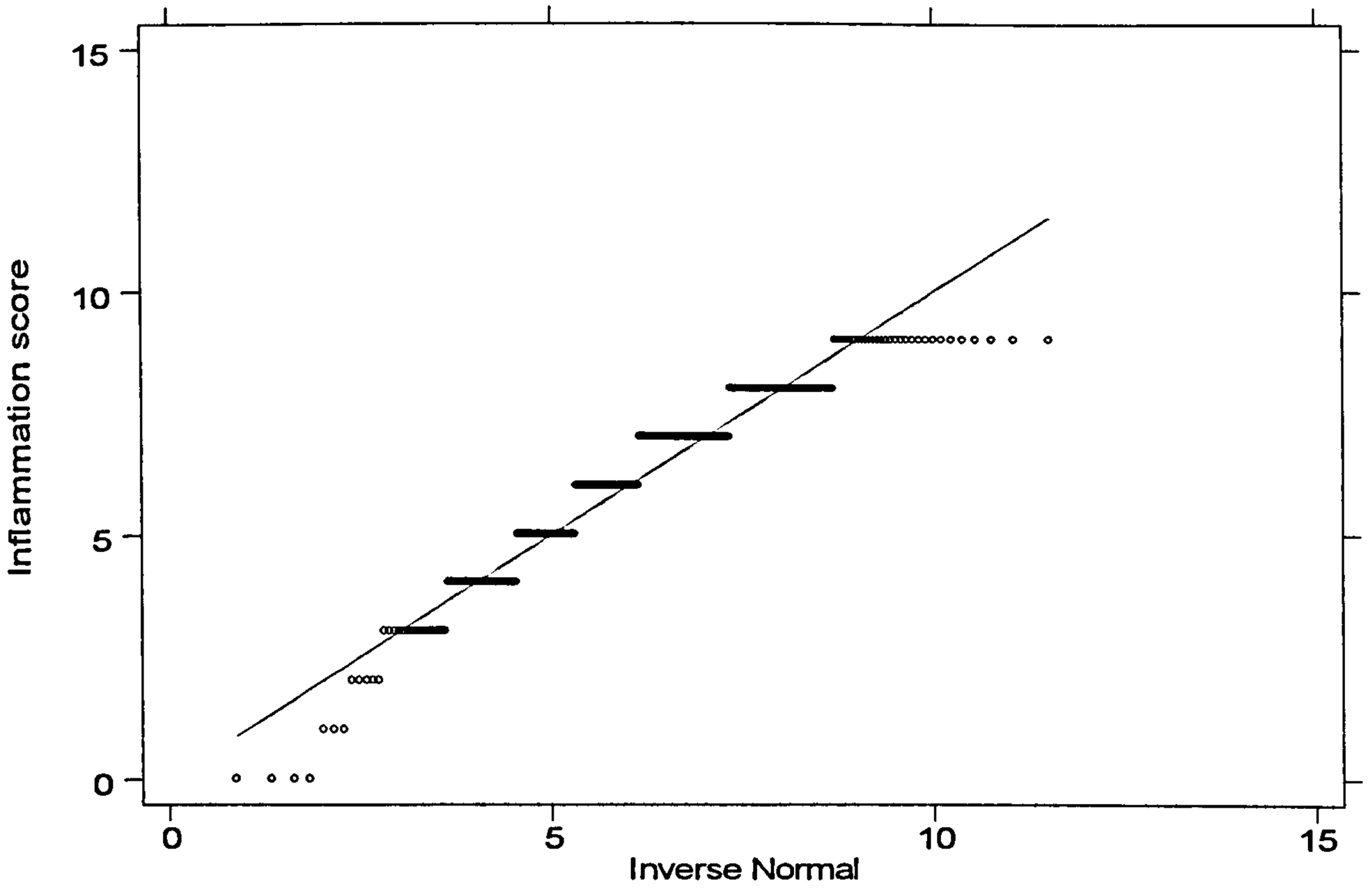



**Figure 7.5: Normal quantile-quantile plot for observation level airway inflammation scores**



## Univariable analyses

As for the pony level analyses, data were examined in the first instance to determine whether there were any statistically significant relationships between outcome scores and different possible explanatory variables. Explanatory variables fell into several discrete groups and took different forms. These included $\log_{10}$ transformed counts (cfu/ml + 1) of total and individual species of bacteria in tracheal washes (continuous), species of bacteria isolated on nasopharyngeal swabs (binary), clinical scores for the same pony from previous or earlier weeks (continuous autoregressive), sex (binary), vaccine group (categorical) and transferrin and protease inhibitor haplotypes (binary).

Linear regression was used to examine separately the relationship of each of the tracheal wash bacterial counts and autoregressive score variables with the aggregated scores. In addition polynomial forms for fitting the data were also evaluated using the FRACPOLY and COMPARE commands available for linear regression analysis in Stata5.0 software. Outcome variables were regressed using the following equation and this was compared with the linear regression result to examine whether it provided statistically significant improvement to the fit of data as measured by deviance reduction based on appropriate degrees of freedom on a $\chi^2$ distribution:

$$\text{outcome } y_{\text{observation}} = \alpha + \beta_1[x_1]^a + \beta_2[x_1]^b \ldots\ldots + \beta_m[x_1]^n$$

where $\alpha$ = intercept estimate

$\beta_1[x_1]^a$ = slope estimate for variable $x_1$ raised to power a

$\beta_2[x_1]^b$ = slope estimate for variable $x_1$ raised to power b

$\beta_m[x_1]^n$ = slope estimate for variable $x_1$ raised to power n

Polynomial regression analyses represented the best fitting regression models containing 2 or more power terms tested from combinations of $x^{-2}$, $x^{-1}$, $x^{-0.5}$, $x^{0.5}$, $x^{1}$, $x^{2}$, $x^{3}$ and $\log_e x$ and including products of $\log_e x$ with all other powers including itself. Higher order models containing 3 or more power terms were also investigated.

Wilcoxon rank sum and Kruskal-Wallis methods were used to examine whether there were any statistically significant differences in aggregated clinical and airway inflammation scores between the categories of binary and categorical explanatory variables. Where significant differences were identified, the direction of the difference in clinical outcome score in relation to the categories of the explanatory variable was examined. All univariable analyses were conducted using Stata5.0 computer software (Stata Corporation).

Ordinary multiple linear regression modelling

Multiple linear and polynomial regression modelling was conducted initially using Stata5.0 computer software (Stata Corporation). These analyses would be able to take account of some of the temporal clustering of observations over relatively short time periods (weeks) by inclusion of autoregressive variables, which for an individual pony related outcome scores in previous weeks to that being modelled. Inclusion of these variables would necessarily reduce the effective sample size and this type of modelling would not account for all the within-pony relationships that may be present.

Model development was conducted in a stepwise manner with the sequential addition of variables that achieved a significance of $P \leq 0.3$ in univariable analysis in descending order of the proportion of the variability ($R^2$ value) in the outcome that they accounted for. Final regression models were those that accounted for the most variability and contained statistically significant variables. Statistical significance was arbitrarily set at the 5% level but as final models were intended to be biologically meaningful, attention was also paid to results that were approaching statistical significant (<10%).

## Multilevel linear regression modelling

Although ordinary linear and polynomial regression modelling included autoregressive variables that would take account of some of the important temporal clustering of these data, these analyses did not take account of the within-pony relationships. Such relationships in repeated observations in ponies might have important implications in the level of significance and estimated values of model parameters. To overcome this, multilevel regression modelling was used in which the hierarchical structure of the data (i.e. observations within ponies) was specified and random effect terms (in addition to the other fixed effect terms) included to model the random variability between ponies and observations. Multilevel regression modelling was conducted using MLwiN1.10 (Multilevel Models Project, Institute of Education; Rasbah *et al.*, 2000), with care taken to ensure that data were correctly ordered with respect to the hierarchical structure prior to analyses.

Model development was conducted in a similar manner to that used for ordinary multiple linear and polynomial regression with sequential addition of variables that achieved a significance of $P \leq 0.3$ in univariable analysis. Improvement in the fit of models of the same sample size was evaluated by the reduction of model deviance and likelihood ratio test. Final models were those in which parameters were either significantly associated with the outcome (Wald $\chi^2$ test $P \leq 0.05$) or the inclusion of which had significantly improved the fit of the model (Likelihood ratio $\chi^2$ test $P \leq 0.05$). All final models included random effect terms to account for variability in the data attributable to both ponies and observations and these were retained even if they were not shown to be statistically significant.

Each multilevel regression model was represented by fixed effect and random effect portions, with the following example for inclusion of a single fixed effect variable ($x_1$):

$$\text{outcome } y_{\text{observation, pony}} = \beta_{0\text{observation, pony}} x_0 + \beta_1 x_{1\text{observation, pony}}$$

where $\beta_{0\text{observation, pony}} = \beta_0 + \mu_{0\text{pony}} + e_{0\text{observation, pony}}$

$x_0$ = constant taking a value of 1

$\beta_1 x_{1\text{observation, pony}}$ = slope estimate for variable $x_{1\text{observation, pony}}$

$\beta_0$ = intercept estimate

$\mu_{0\text{pony}}$ = pony-level random coefficient (mean = 0, variance = $\sigma^2_{\mu 1}$)

$e_{0\text{observation, pony}}$ = observation-level random coefficient (mean = 0, variance = $\sigma^2_{e0}$)

The fixed effects are represented by the relevant β estimates and their standard errors (S.E.) for the intercept ($\beta_0$) and slopes ($\beta_1$) for the explanatory variable(s) included in the model. The random effects are represented by variance estimates at each hierarchical level within the data. The hierarchical structure of these data was repeated observations (known as level 1 in the hierarchy) clustered within each pony (level 2). This meant that the level 2 random effects represented the variability between different ponies (pony level random departures or residuals, $\mu_{0\text{ pony}}$, were normally distributed with mean of zero and variance $\sigma^2_{\mu 0}$). The level 1 random effects represented the variability between individual observations within ponies (observation level random departures or residuals, $e_{0\text{ observation, pony}}$, were normally distributed with mean of zero and variance $\sigma^2_{e0}$). It was possible that the random variability between ponies might have been best modelled by differences in both their intercepts and their slopes (so called random intercept and slope models) or it may have been more appropriate to only model variation in intercepts (random intercept only models).

The intra-pony correlation, which was the proportion (%) of the total variance of observations and ponies attributable to variation between ponies, was also calculated for individual multilevel models:

$$\text{Intra-pony correlation (\%)} = \frac{\text{Level 2 (pony-level) variance}}{\text{Level 1 (observation-level) variance + Level 2 (pony-level) variance}} \times 100$$

Therefore, the more similar ponies were to each other, as measured by a lower level 2 variance, then the lower the intra-pony correlation.

After final models were derived, the fits to the data were investigated by examination of residual values at different levels within the data. Ranking and plotting of residual values and their 95% confidence intervals for data at both pony and observation levels, in so-called 'caterpillar plots', allowed the identification of animals, and observations from these individuals, whose outcomes were poorly predicted by the model in question. Models were also re-analysed with specified poorly fitting observations or ponies (large value residuals) fitted with separate intercepts (and slopes if necessary) in the fixed effect part of the model and not contributing to the estimation of the random effects.

*7.3.2.2 Individual clinical signs*

As described previously, each pony had a weekly binary outcome for each of the 5 individual clinical parameters of nasal discharge, ocular discharge, coughing, abnormal breathing and submandibular lymph node enlargement.

Univariable analyses

Data were initially examined for the univariable association of the probability of presence of each individual clinical sign with explanatory variables (same variables as for

aggregated clinical sign and airway inflammation scores) by inclusion in a simple ordinary logistic regression (OLR) model.

$$\text{logit}\,(p_{observation}) = \log_e[p_{observation}/(1-p_{observation})] = \alpha + \beta_1 x_{1observation}$$

Where the logit of probability ($p_{observation,}$) is the natural logarithm of the odds of the binary outcome being observed ($[p_{observation}/(1-p_{observation})]$), $\alpha$ is an estimate of the population intercept and $\beta_1$ is the slope estimate for variable $x_{1observation}$.

The association between outcome and each explanatory variable was expressed as a beta estimate and standard error ($\beta$ and S.E. $\beta$), an estimated unadjusted (crude) OR with 95% confidence intervals around the estimate and corresponding Wald $\chi^2$ P-value.

The risks of each outcome with $\log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes were examined graphically to assess the most suitable way to express the levels of this variable in further multivariable analyses. Where there was not a satisfactory linear relationship between the logit of outcome risk and $\log_{10}$ cfu/ml *S. zooepidemicus*, the square and cube of $\log_{10}$ cfu/ml *S. zooepidemicus* were examined for their suitability of fitting data by inclusion as quadratic (i.e. $\log_{10}$ cfu/ml and $[\log_{10}$ cfu/ml$]^2$ included as model terms) or cubic (i.e. $\log_{10}$ cfu/ml and $[\log_{10}$ cfu/ml$]^3$ included) model terms. If these terms were also considered unsuitable for modelling the risk of outcome with $\log_{10}$ cfu/ml *S. zooepidemicus* then an appropriate categorical summary of the data was chosen.

Multivariable logistic regression analyses ignoring pony-level random effects

As for analyses for the aggregated clinical sign and airway inflammation scores, multivariable analyses of individual binary clinical outcomes were conducted initially using logistic regression with inclusion of autoregressive variables. This would take account of

some of the temporal associations between repeated observations in the data but would not account for the within-pony relationships.

A forward stepwise approach was adopted with systematic addition of explanatory variables that achieved a significance of P≤0.3 in univariable analysis and in descending order of their strength of association with the outcome. Variables were only retained if they were associated with disease (Wald $\chi^2$: P ≤ 0.05) or if their inclusion resulted in a significant improvement in model fit as measured by the likelihood ratio statistic (LRS $\chi^2$: P ≤ 0.05) (Hosmer & Lemeshow, 1989). To ensure that statistically significant associations had not been inadvertently rejected, the final parsimonious model was checked by systematic addition of rejected variables one at a time.

Statistical interaction was examined by the addition of biologically meaningful, 2-way interaction terms between main effect variables. Interaction terms that provided a significant improvement to model fit as measured by likelihood ratio statistic (LRS $\chi^2$: P ≤ 0.05) were retained. Multivariable logistic regression analyses were conducted using the 'Logistic regression' analysis option in Egret software (Cytel Software Corporation).

Multivariable logistic regression analyses accounting for pony-level random effects

In order to account for the clustering of repeated observations within individual ponies in these data, 2 separate software packages were used and compared to conduct multivariable logistic regression modelling that included specific terms for random variability between ponies (pony-level random effects).

Egret software (Cytel Software Corporation), which provides maximum likelihood estimates for regression parameter estimates, was used to extend the multivariable models produced with no account taken of random variation between ponies. Logistic regression models were extended by inclusion of a random effects term matched on pony using the 'Logistic regression with distinguishable binomial random effect' analysis option.

$$\text{logit}\,(p_{\text{observation, pony}}) = \log_e[p_{\text{observation, pony}}/(1-p_{\text{observation, pony}})] + \sigma_{\text{pony}}u$$

where $\log_e[p_{\text{observation,pony}}/(1-p_{\text{observation,pony}})] + \sigma_{\text{pony}}u = \alpha + \beta_1 x_{1\text{observation, pony}} + \sigma_{\text{pony}}u$

Where the logit of probability ($p_{\text{observation, pony}}$) is the sum of the natural logarithm of the odds of the binary outcome being observed ($[p_{\text{observation, pony}}/(1-p_{\text{observation, pony}})]$) and the random variable $\sigma_{\text{pony}}u$, which has a mean of zero and variance of one. The term $\sigma_{\text{pony}}$ is a positive scalar (expressed as %SCL in EGRET output) equivalent to the coefficient of the pony (group) effect and u is a standardised binomial random variable which is always equal to one. The term $\alpha$ is an intercept term (expressed as %GM in Egret output) and $\beta_1$ is the slope estimate for variable $x_{1\text{observation, pony}}$.

Results were presented as beta estimates and standard errors ($\beta$ & S.E. $\beta$), estimated adjusted OR with 95% confidence intervals around the estimates and corresponding Wald $\chi^2$ P-values. Due to the lack of approximation of differences in deviances between models with and without a random effect term to a chi-squared distribution and consequent difficulty in their interpretation, formal likelihood ratio statistics and corresponding P-values were not presented.

MLwiN1.10 (Multilevel Models Project, Institute of Education) software was also used to examine factors associated with presence of individual clinical signs. As for multilevel linear regression modelling, the hierarchical structure of observations within individual ponies was specified and model building was conducted in a forward stepwise manner similar to that used for ordinary multivariable logistic regression.

$$\text{outcome } y_{\text{observation, pony}} = \pi_{\text{observation, pony}} + e_{0\text{observation, pony}}x_0$$

where $\text{logit}\,(\pi_{\text{observation, pony}}) = \beta_{1\text{pony}}x_1 + \beta_2 x_{2\text{observation, pony}}$

$e_{0\text{observation, pony}}$ = observation-level random coefficient (mean = 0, variance = 1)

$$x_0 = 1*[\pi_{\text{observation, pony}}(1-\pi_{\text{observation, pony}})/n_{\text{observation, pony}}]^{0.5}$$

$\beta_{1\text{pony}}x_1 = \beta_1 + \mu_{1\text{pony}}$

$\beta_2 x_{2\text{observation, pony}}$ = slope estimate for variable $x_{2\text{observation, pony}}$

$\beta_1$ = intercept estimate

$x_1$ = constant taking a value of 1

$\mu_{1\text{pony}}$ = pony-level random coefficient (mean = 0, variance = $\sigma^2_{\mu 1}$)

Modelling was conducted using the second order penalised quasilikelihood (PQL) estimation technique with model estimates derived by both iterative generalised least squares (IGLS) and the less biased restricted iterative generalised least squares (RIGLS) methods. The results from these 2 methods were compared with those produced for the maximum likelihood estimation techniques of Egret software. As previously, the fit of final models was investigated by the examination of residual values, particularly at the level of individual ponies within the data.

# CHAPTER 8

# RESULTS

## 8.1 Description of pony level data

Table A2.1 (Appendix 2) summarises details of pony level explanatory variables and cumulative summary outcome measures for each of the 29 ponies grouped according to their vaccine status along with details of whether they possessed D and/or F2 transferrin haplotypes. The cumulative summary outcome measures are the sum of weekly average scores for individual signs of nasal and ocular discharges, cough, abnormal breathing/dyspnoea and SMLN enlargement over the study period. Clinical and CDNS scores are the sum of the cumulative scores of each of the individual signs including and excluding ocular discharge, respectively. Results of the summary statistics (mean, S.D., S.E. and median; bottom 4 rows of Table A2.1) show that coughing was the least frequently observed individual clinical sign, with some animals not being observed to cough at all during the period of the study.

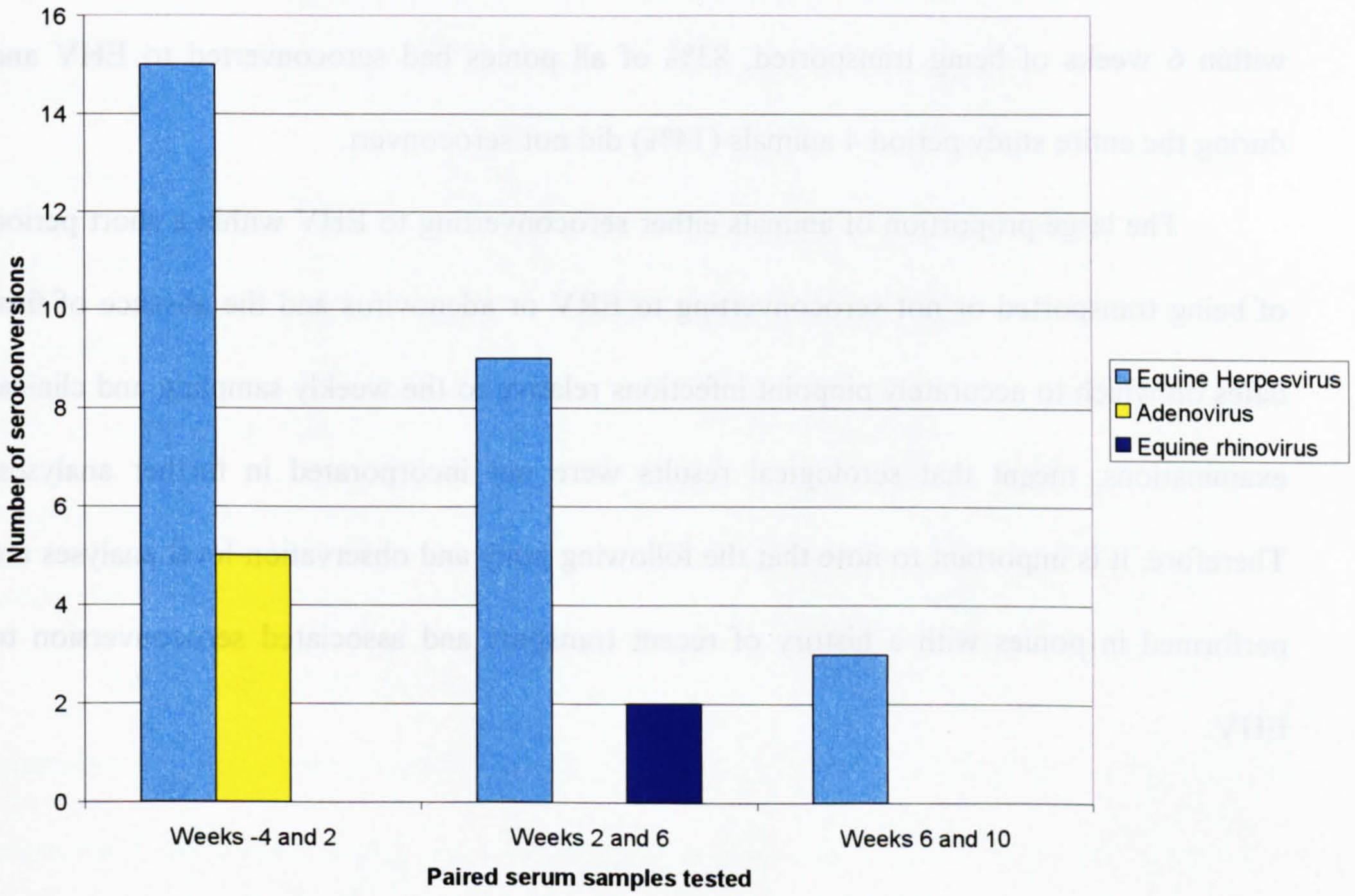
Tables A2.2a - e summarises details of pony level explanatory variables and cumulative summary measures ($log_{10}$ total cfu/ml, maximum $log_{10}$ cfu/ml, mean $log_{10}$ cfu/ml, total weeks with $\geq 10^3$, $\geq 10^4$ and $\geq 10^5$ cfu/ml and $log_{10}$ cfu/ml at week 26) for specific bacterial infections in tracheal wash samples, including *S. zooepidemicus*, *A. equuli*, *Pasteurella* spp., *B. bronchiseptica* and non-haemolytic *Streptococcus* spp., respectively. Results also include summary statistics for each cumulative parameter and demonstrate that *A. equuli* was the least prevalent infection in tracheal washes with respect to the quantities of bacteria isolated from tracheal washes.

### 8.1.1 Serology results

Serology results were not available for all observations but were taken at approximately monthly intervals during the study. The first sample was taken immediately prior to vaccination (week –4) and subsequent samples were collected at weeks 2, 6 and 10 after transportation to Newmarket.

Although there was no serological evidence of influenza virus infection during the study there were seroconversions to other equine viral infections. Figure 8.1 summarises the number of seroconversions detected to EHV, equine adenovirus and ERV for each of 3 sets of paired sera (i.e. weeks –4 and 2, weeks 2 and 6 and weeks 6 and 10).

**Figure 8.1: Summary of numbers of seroconversions to EHV, equine adenovirus and ERV for different paired sera**



The 5 ponies in the late introduction group were not available for blood sampling at the time of vaccination and so the first set of paired sera (weeks –4 and 2) only apply to the 24 vaccine and placebo administered ponies. The 2 subsequent sets of paired sera (weeks 2 and 6 and weeks 6 and 10) apply to all 29 ponies.

There were only 2 ponies that seroconverted to ERV and both of these occurred between weeks 2 and 6 and 5 ponies seroconverted to equine adenovirus, all between weeks –4 and 2. Of the 24 ponies with paired sera available between weeks –4 and 2, 15 (62.5%) seroconverted to EHV. Of the 14 ponies with samples available between weeks 2 and 6 which had either not seroconverted earlier (n=9) or were late introductions (n=5), 9 (64.3%) seroconverted to EHV, with 4 of these being late introductions. There were 3 seroconversions to EHV between weeks 6 and 10. These included 2 ponies that had seroconverted previously between weeks –4 and 2 and the remaining late introduction pony that had not seroconverted between weeks 2 and 6. These serological results show that within 6 weeks of being transported, 83% of all ponies had seroconverted to EHV and during the entire study period 4 animals (14%) did not seroconvert.

The large proportion of animals either seroconverting to EHV within a short period of being transported or not seroconverting to ERV or adenovirus and the absence of firm dates on which to accurately pinpoint infections relative to the weekly sampling and clinical examinations, meant that serological results were not incorporated in further analyses. Therefore, it is important to note that the following pony and observation-level analyses are performed in ponies with a history of recent transport and associated seroconversion to EHV.

## 8.2 Pony level analyses

### 8.2.1 Relationship between clinical disease scores and pony level explanatory variables

Table A2.3 summarises the results of non-parametric tests to examine whether there were significant differences in clinical disease scores between the pony level variables of sex, vaccine group and each of the transferrin and protease inhibitor haplotypes.

It can be seen that overall there were no statistically significant differences between any of the individual or aggregated clinical parameters and the sexes, vaccine groups or those ponies with and without any of the protease inhibitor haplotypes. However, between the sexes there were differences in scores for CDNS, nasal discharge and abnormal breathing/dyspnoea that approached statistical significance ($0.05<P<0.1$). For all 3 of these parameters female ponies had higher scores than expected and males had lower scores, i.e. there was consistency in the direction of this effect.

Among 2 of the transferrin haplotypes (D and F2) there were consistently statistically significant differences in clinical scores between ponies that possessed particular haplotypes and those that did not. The 18 ponies that had transferrin haplotype D ranked significantly lower scores for both aggregated clinical parameters and for the abnormal breathing/dyspnoea score, compared to the 11 ponies that did not possess this haplotype. The 14 ponies that had transferrin haplotype F2 ranked significantly higher scores for both aggregated clinical parameter scores as well as for the scores for nasal discharge, cough and abnormal breathing/dyspnoea, compared with 15 ponies not possessing this haplotype.

Table A2.4 gives specific details of data ordered by increasing measures of each cumulative aggregated and individual clinical sign score for individual horses. Statistically significant differences using the non-parametric Wilcoxon ranked sum test were found between rankings of clinical, CDNS, nasal discharge, cough and abnormal breathing/dyspnoea scores between ponies that did and did not possess transferrin D (without F2 as a DF2 phenotype) or transferrin F2 haplotypes. Ponies with the transferrin

F2 haplotype (including DF2 phenotypes) have been highlighted. It can be seen that for clinical, CDNS, nasal discharge, cough and abnormal breathing/dyspnoea scores where there was a statistically significant difference between transferrin haplotypes, the majority of F2 ponies appear in the lower halves of the tables. This indicated that ponies possessing the F2 haplotype suffered cumulatively more signs over the study period than those that did not. All the other ponies except pony 23 possessed the transferrin D haplotype as either a homozygote or as a heterozygote and this accounted for significant differences between ponies with and without the D haplotype.

Four ponies possessed both the D and F2 haplotypes (i.e. DF2 phenotypes) and examination of raw clinical score data (Tables A2.4) suggested that these individuals were more like most F2 haplotypes than D haplotypes, i.e. they tended to have higher clinical parameter scores. Therefore, further analyses were conducted to examine the effect of the transferrin D haplotype on clinical parameters in the absence of possible dominance from the F2 haplotype. Analyses were repeated with exclusion of the 4 DF2 phenotype ponies. When these ponies were excluded from the analyses there was now a statistically significant difference in scores for nasal discharge (P=0.04) and cough (P=0.02) where previously these were not significant, with horses possessing the D haplotype having lower scores. In addition, exclusion of the 4 DF2 phenotype ponies from the earlier analyses that had yielded statistically significant results, all resulted in lower P-values. The changes in P-values were for clinical score, P=0.002 from P=0.01; CDNS score, P=0.003 from P=0.02 and abnormal breathing/dyspnoea score, P=0.0003 from 0.008.

Additional analyses were also conducted to examine whether inclusion in the analyses of the 5 non-vaccinated ponies from other parts of Wales (Late intro group) had an effect on the strong association between clinical disease and D and F2 transferrin haplotypes. Analyses were repeated excluding these 5 ponies. Results showed that the relationship between transferrin groups D and F2 and clinical outcomes remained

statistically significant and in the same direction when only the vaccinated and placebo administered control ponies (n=24) were included in the analyses. The lack of apparent effect of vaccine group in this and earlier analyses, was taken to justify that data from all 29 ponies be included in all analyses.

These results indicated that clinical scores varied most significantly with the transferrin haplotypes of D and F2 but there was also some evidence of variation according to sex. In order to investigate whether the variation in scores between sexes was explained by the effect of transferrin group or *vice versa*, a stratified analysis was conducted. In this stratified approach, the effect of sex for each category of transferrin F2 haplotype (present and absent) and the effect of F2 for each sex category (male and female) were examined. Data for the 3 outcome variables of CDNS score, nasal discharge score and abnormal breathing/dyspnoea score, which were all approaching statistical significance for the effect of sex were used in the stratified analysis. Results for the stratified analyses are summarised in Table 8.1.

**Table 8.1: Summary of non-parametric analyses examining differences in clinical scores according to sex and transferrin F2 haplotype stratified by levels of the other category**

| Outcome variable | Explanatory variable by stratification variable | Stratum | Total n | P-value |
|---|---|---|---|---|
| CDNS score | Sex by transferrin F2 | F2 negative | 15 | 0.175 |
| | | F2 positive | 14 | 0.386 |
| | **Transferrin F2 by sex** | **male** | **14** | **0.072** |
| | | **female** | **15** | **0.013** |
| Nasal discharge score | Sex by transferrin F2 | F2 negative | 15 | 0.156 |
| | | F2 positive | 14 | 0.257 |
| | **Transferrin F2 by sex** | **male** | **14** | **0.095** |
| | | **female** | **15** | **0.025** |
| Abnormal breathing | Sex by transferrin F2 | F2 negative | 15 | 0.622 |
| | | F2 positive | 14 | 0.201 |
| | **Transferrin F2 by sex** | **male** | **14** | **0.056** |
| | | **female** | **15** | **0.002** |

Results indicated that overall there was still an approaching significant difference ($P<0.1$ with sample size reduced by ~50% in each stratum) in clinical outcome scores for all 3 tested variables between those ponies with and without transferrin F2 haplotype even when tested for (i.e. stratified by) each sex separately. In contrast, when stratified by transferrin F2 haplotype, there was no longer any evidence for a significant difference in clinical score ranking between the 2 sexes. Examination of the distribution of transferrin haplotypes between the sexes explained this result if the true explanation was transferrin haplotype. 9 females possessed haplotype F2 compared with only 5 males, and 6 females possessed haplotype D in the absence of F2 compared with 8 males.

*8.2.1.1 Summary*

In conclusion, the results of these analyses using various different individual and aggregated clinical outcome measures suggested that there was a difference in the outward demonstration of clinical respiratory disease between horses possessing particular transferrin haplotypes. This was consistent with a genetically based variation in susceptibility to clinical respiratory disease in equids.

In these 29 ponies monitored intensively and repeatedly over a 10 week period, the 14 ponies that possessed homozygote or non-F2 heterozygote transferrin D haplotype demonstrated statistically significantly less cumulative clinical disease as measured by a sum of clinical scores and analysed using non-parametric ranking techniques. Using the same techniques, 14 ponies possessing the F2 transferrin haplotype and including 4 DF2 phenotype ponies, demonstrated statistically significantly more cumulative clinical disease.

### 8.2.2 Relationship between clinical disease scores and infectious summary variables

Tables A2.5 - A2.9 summarise the results of linear regression analyses conducted to assess any linear relationship between different cumulative clinical parameter scores and

different pony level infectious summary variables for tracheal isolations. The tables (A2.5 - A2.9) are for *S. zooepidemicus*, *A. equuli*, *Pasteurella* spp., *B. bronchiseptica* and non-haemolytic *Streptococcus* spp., respectively. For each analysis there were a total of 29 observations corresponding to cumulative summary measures for each individual pony for the period of the study. In each regression, the relevant clinical score was regressed on a single infection variable.

*8.2.2.1 S. zooepidemicus*

The results of the regression analyses show that for most of the summary measures for *S. zooepidemicus* in tracheal washes (except $\log_{10}$ cfu/ml count at week 26), there was a statistically significant positive linear relationship between increasing infectious burden and increasing clinical parameter scores (except ocular discharge). In general this indicated that the higher the overall clinical score that ponies exhibited the more likely they were to have higher burdens of *S. zooepidemicus* infection, which was measured in a variety of ways (e.g. $\log_{10}$ total cfu/ml, maximum or mean $\log_{10}$ cfu/ml or number of weeks with $>\log_{10}$ cfu/ml). This rather crude analysis simply tells us that the more severely affected horses tended to have greater overall burdens of *S. zooepidemicus* in their tracheas and for longer periods but does not reveal very much about the dynamics or distribution of infection over the course of the study period.

For illustration, Figures A2.1 - A2.5 show the distribution of cumulative CDNS scores and various corresponding *S. zooepidemicus* parameter measures, with points identified as to the ponies' transferrin D and F2 haplotype status. Graphs for the total week count parameters were plotted incorporating 5% spherical random noise around plotted points to avoid loss of information with overlaying of points. This was particularly important for graphs plotting CDNS score with autoregressive scores. It can be clearly seen that the majority of ponies with transferrin D haplotype (but without F2 haplotype) occupy

the lower points to the left and those with F2 haplotype (including DF2 phenotypes) occupy the higher points to the right.

*8.2.2.2 A. equuli*

There was evidence for a positive linear relationship between several summary measures of tracheal *A. equuli* infection and clinical parameter scores, although involving fewer parameters than had been the case for *S. zooepidemicus*. The aggregated parameters of clinical score and CDNS score as well as cough and abnormal breathing scores demonstrated a statistically significant association with $\log_{10}$ total cfu/ml and maximum $\log_{10}$ cfu/ml and total weeks with $\geq 10^5$ cfu/ml.

*8.2.2.3 Pasteurella spp.*

There was evidence for a positive linear relationship between several summary measures of tracheal *Pasteurella* spp. infection and clinical parameter scores, although again involving fewer parameters than had been the case for *S. zooepidemicus*. The aggregated parameters of clinical score and CDNS score as well as nasal discharge and abnormal breathing scores demonstrated a statistically significant association with $\log_{10}$ total cfu/ml and maximum $\log_{10}$ cfu/ml and total weeks with $\geq 10^4$ and $\geq 10^5$ cfu/ml.

*8.2.2.4 B. bronchiseptica*

In the summary data examined here there were no obviously demonstrable overall linear relationships between summary measures of tracheal *B. bronchiseptica* infection and any biologically meaningful cumulative clinical parameter scores. Analyses for the relationship between the $\log_{10}$ cfu/ml tracheal wash count of *B. bronchiseptica* at week 26 and each outcome measure were not possible as *B. bronchiseptica* was not isolated from any tracheal washes at this point in the study.

*8.2.2.5 Non-haemolytic Streptococcus spp.*

There was evidence for a negative linear relationship between several summary measures of tracheal non-haemolytic *Streptococcus* spp. infection and clinical parameter scores, although this involved fewer parameters than had been the case for *S. zooepidemicus*. The aggregated parameters of clinical score and CDNS score and nasal discharge and abnormal breathing scores demonstrated a statistically significant inverse association with both geometric mean $\log_{10}$ cfu/ml and total weeks with $\geq 10^3$ cfu/ml and SMLN score was inversely associated with the latter. In addition, nasal discharge and SMLN scores were also significantly negatively associated with the count of non-haemolytic *Streptococcus* spp. in tracheal washes sampled at week 26.

*8.2.2.6 Summary*

In conclusion, results indicated that at the pony level there were statistically significant positive linear relationships between various cumulative clinical parameter scores and summary measures of infectious burden in the trachea for *S. zooepidemicus, A. equuli* and *Pasteurella* spp. and negative ones for non-haemolytic *Streptococcus* spp..

### 8.2.3 Relationship between infectious summary variables and sex, vaccine group, transferrin and protease inhibitor haplotypes

Tables A2.10a/b - A2.14 (Appendix 2) summarise the results of non-parametric tests to examine whether there were significant differences in infectious summary measures between the pony level variables of sex, vaccine group and transferrin and protease inhibitor haplotypes for *S. zooepidemicus, A. equuli, Pasteurella* spp., *B. bronchiseptica* and non-haemolytic *Streptococcus* spp. respectively.

*8.2.3.1 S. zooepidemicus*

It can be seen that overall there was no statistically significant differences in any of the summary parameters for tracheal *S. zooepidemicus* between the vaccine groups or those ponies with and without any of the protease inhibitor haplotypes. However, between the sexes there were differences in total, maximum, mean $log_{10}$ cfu/ml and total weeks with $\geq 10^4$ cfu/ml scores that approached statistical significance ($0.05<P<0.1$). For all these parameters female ponies had higher rank sum scores than expected and males had lower rank sum scores, i.e. there was consistency in the direction of this effect. However, as previously explained the distribution of D and F2 haplotypes between the sexes was sufficient to provide an alternative explanation for the strength of association with sex.

Among 2 of the transferrin haplotypes (D and F2) there was consistency in statistically significant differences in tracheal *S. zooepidemicus* summary measures between ponies that possessed particular haplotypes and those that did not (Tables A2.10a/b). In the analyses for transferrin D haplotype, results are presented with the 4 ponies that possessed DF2 phenotype excluded from the analyses. The 14 ponies that had transferrin haplotype D in the absence of F2 ranked significantly lower scores for total, maximum, mean $log_{10}$ cfu/ml and total weeks with $\geq 10^4$ and $\geq 10^5$ cfu/ml, compared to the 11 ponies that did not possess this haplotype. The 14 ponies that had transferrin haplotype F2 ranked significantly higher scores for these measures, compared with 15 ponies not possessing this haplotype. Table A2.10b summarises the relative distribution of transferrin D and F2 haplotypes in ascending order of magnitude of various cumulative measures of tracheal *S. zooepidemicus* infection.

Given the relationship between bacteria and clinical scores, these results were consistent with the earlier findings that transferrin haplotypes D and F2 were associated with significant differences in cumulative clinical parameter scores. In addition, the direction of the ranking scores were also consistent and biologically meaningful i.e. that transferrin D haplotypes not heterozygous with F2 resulted in both lower clinical scores and infectious

parameter measures and possession of F2 haplotype, even if heterozygous with D, gave higher scores.

*8.2.3.2 A. equuli*

There was no evidence from these data for any significant association between the different tracheal *A. equuli* summary outcome measures and any of the pony level variables examined.

*8.2.3.3 Pasteurella spp.*

There was some consistency in statistically significant differences in tracheal *Pasteurella* spp. summary measures between ponies that possessed transferrin haplotype O and those that did not. The 9 ponies that possessed transferrin haplotype O ranked statistically significantly lower scores for $\log_{10}$ total cfu/ml, maximum $\log_{10}$ cfu/ml, mean $\log_{10}$ cfu/ml and total weeks with $\geq 10^3$ and $\geq 10^4$ cfu/ml, compared to the 20 ponies that did not possess this haplotype.

*8.2.3.4 B. bronchiseptica*

There was no evidence from these data for any significant association between different tracheal *B. bronchiseptica* summary outcome measures and any of the pony level variables examined.

*8.2.3.5 Non-haemolytic Streptococcus spp.*

There was some consistency in statistically significant differences in tracheal non-haemolytic *Streptococcus* spp. summary measures between ponies that possessed either transferrin F2 or O haplotypes and those that did not and between different vaccine groups. The 14 ponies that possessed transferrin haplotype F2 ranked statistically significantly lower scores for mean $\log_{10}$ cfu/ml and total weeks with $\geq 10^3$ and $\geq 10^4$ cfu/ml, compared to the 15

ponies that did not possess this haplotype. Similarly the 9 ponies that possessed transferrin haplotype O ranked statistically significantly higher scores for the same 3 parameters compared to the 20 ponies that did not possess this haplotype. There was evidence that the 5 non-vaccinated ponies that were introduced later had statistically significantly lower scores for mean $\log_{10}$ cfu/ml and total weeks with $\geq 10^3$ cfu/ml. These results, when taken together with the results for *S. zooepidemicus*, are consistent with an inverse reciprocal relationship between non-haemolytic *Streptococcus* spp. and *S. zooepidemicus* in tracheal washes. That is factors such as transferin F2 haplotype or increased clinical scores that were positively associated with different measures of *S. zooepidemicus* infection in tracheal washes, were inversely associated with some measures of non-haemolytic *Streptococcus* spp. in the same washes. This is consistent with the findings of the case control study of clinically apparent respiratory disease in Thoroughbred racehorses (Section 2), and as with that study, it is not clear whether this is biologically meaningful or whether it is due to some artificial sampling error that results in reduced detection of bacteria such as non-haemolytic *Streptococci* in the presence of larger numbers of presumptively pathogenic bacteria.

*8.2.3.6 Summary*

The results of these analyses using various different tracheal *S. zooepidemicus*, *Pasteurella* spp. and non-haemolytic *Streptococcus* spp. summary outcome measures suggest that there was a difference in the overall infectious burden suffered by horses possessing particular transferrin haplotypes. This may be consistent with a genetically based variation in susceptibility to bacterial respiratory infection in horses. It may be no surprise that different organisms demonstrate significant differences according to whether ponies possess different transferrin haplotypes as this may reflect differences in the mechanisms of acquisition of iron by the different bacterial species. For species of bacteria possessing transferrin binding proteins (TBPs) such as *Haemophilus*, *Actinobacillus* and *Pasteurella*

spp., transferrin is a recognised source of iron with mechanisms well described for its acquisition. To date, no mechanism for iron acquisition from transferrin has been recognised for Streptococci. Therefore, the findings for Streptococci in this study may be consistent with either linkage to some other disease susceptibility factor, acquisition of iron from particular transferrin haplotypes or use of transferrin for some other purpose.

### 8.2.4 Preliminary discussion of findings and conclusions from pony level analyses

Data in this study of naturally occurring respiratory disease in recently weaned Welsh Mountain ponies were relatively complete and consistent compared to many field studies. Detailed clinical and infectious data were collected at regular intervals during the study from all 29 study subjects (ponies). Certain variables including sex, vaccine group and transferrin and protease inhibitor haplotypes were available for each pony and these were set at the pony level. Twice weekly clinical observations were averaged so as to correspond to weekly infectious sampling data. These were observation level data and were clustered within individual ponies.

To examine preliminary univariable associations between parameters at the pony level, summary measures were generated for clinical outcome and infectious parameters. There was no evidence for a statistically significant effect on any aggregated or individual clinical summary parameters according to vaccine group. There was some limited evidence for a statistically significant effect on some aggregated or individual clinical summary parameters according to sex. There was evidence for statistically significant differences in various aggregated and individual clinical summary parameters between ponies possessing either the D or F2 transferrin haplotypes. Ponies with the D haplotype in the absence of F2 suffered significantly less severe disease whereas ponies with F2 suffered more severe disease. Stratification of this small dataset to examine whether sex or transferrin group were

associated with differences in clinical parameters, suggested that this was due to transferrin haplotype.

At the pony level there was evidence that individuals that had higher overall clinical disease scores tended to have higher overall infectious burdens. This was most marked for *S. zooepidemicus* and less marked for *A. equuli* and *Pasteurella* spp. and was not evident for *B. bronchiseptica.* There was some evidence for a negative relationship between non-haemolytic *Streptococcus* spp. in tracheal washes and overall clinical disease scores.

There was evidence for statistically significant differences in various summary measures for tracheal *S. zooepidemicus* infection between ponies possessing either the D or F2 transferrin haplotypes. Ponies with the D haplotype in the absence of F2 had significantly less infectious burden whereas ponies with F2 had greater burdens. There was also evidence for significant differences in burden of tracheal *Pasteurella* spp. infection between ponies with and without transferrin O haplotype. Ponies with the O haplotype had significantly less infectious burden than those without it and this may be consistent with iron from transferrin O being less usable by *Pasteurella* spp..

Caution is required in interpretating the multiple statistical comparisons performed in these pony-level analyses (e.g. Table A2.3) because as the number of comparisons increases the possibility of a chance occurrence of a 'statistically significant' result similarly goes up. There is currently much debate as to whether such results should be automatically accompanied by some form of correction, such as using Bonferroni's method, to help in identifying true rather than chance occurrences (Perneger, 1998). Such corrections have not been applied here, although I am aware of the issues surrounding such multiple comparisons. I would argue that in considering consistency of results for different outcomes and an unwillingness to over interpret occasionally significant results I have imposed 'correction', whilst at the same time have not stuck to a strict corrected P-value paradigm.

In conclusion, these data preliminarily suggest that there might be a genetic basis for variation in susceptibility to clinical respiratory disease. This may be manifested by a variability in susceptibility to infection with bacteria such as *S. zooepidemicus* and *Pasteurella* spp., which have been shown to be significantly associated with both clinical and subclinical (IAD) respiratory disease.

The lack of association between cumulative ocular discharge score and any of the explanatory variables that were examined suggested that this was not a useful sign with which to evaluate clinical severity of respiratory disease. This was confirmed by the increased statistical significance of associations between cumulative CDNS score (which did not include ocular discharge) and the vast majority of explanatory variables compared with their associations with cumulative clinical score (which did include ocular discharge).

## 8.3 Description of observation level data

Figure A2.6 summaries the average weekly scores for individual clinical signs (different shading/colour for each sign) for each individual pony in a graphical format as stacked bars, with the peak of the stacked bar corresponding to the overall average clinical score for that week. Each graph also represents the $\log_{10}$ colony forming units per ml of tracheal wash of different bacterial species, with *A. equuli* and *Pasteurella* spp. being combined and referred to as *Pasteurella*-like spp..

## 8.4 Observation level analyses

### 8.4.1 Aggregated clinical and airway inflammation scores

#### *8.4.1.1 Univariable analyses*

Table A2.15 summarises the results and comparisons of linear and best fitting polynomial regression analyses of clinical, CDNS and airway inflammation scores with $\log_{10}$ cfu/ml for total bacterial and species specific tracheal wash counts and autoregressive

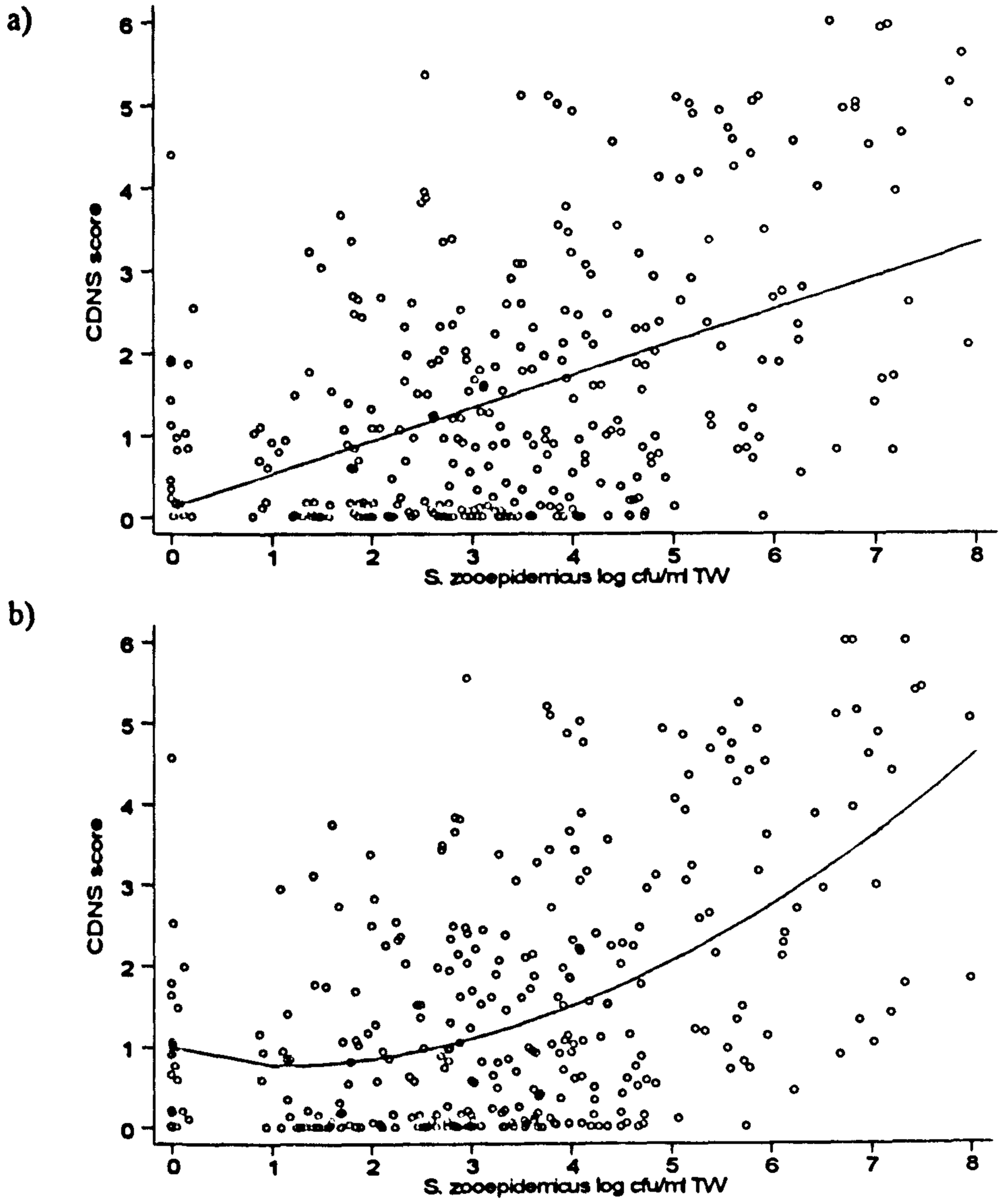
variable scores. The results of polynomial regression analyses represented the best fitting regression models containing 2 power terms tested from combinations of $x^{-2}$, $x^{-1}$, $x^{-0.5}$, $x^{0.5}$, $x^{1}$, $x^{2}$, $x^{3}$ and $\log_e x$ and including products of $\log_e x$ with all other powers including itself. Higher order models containing 3 or more power terms were investigated but found not to significantly improve the fit of the data.

Comparison of the respective linear and polynomial model $R^2$ values showed that in all cases the fitting of a 2-power polynomial regression model increased the amount of variability in the data explained by the models. The last 2 columns of Table A2.15 represented the decrease in deviance by the polynomial model compared to the linear regression and the corresponding P-value based on 3 degrees of freedom in a chi-squared distribution. This indicated whether the polynomial model provided a significantly improved fit of the data compared to the linear model.

Results indicated that there was no evidence of a significant linear relationship between increasing scores of each of the 3 outcome measures and $\log_{10}$ cfu/ml counts of *A. equuli* and *B. bronchiseptica* and use of a best fitting, 2-power term polynomial regression model did not provide significant improvement.

Results indicated that there was a significant linear relationship between increasing score for all 3 outcome measures and $\log_{10}$ cfu/ml *S. zooepidemicus* and use of a best fitting, 2-power term polynomial regression model only provided significant improvement over a linear model for CDNS score. Figure 8.2 illustrates the distribution of CDNS scores versus corresponding $\log_{10}$ cfu/ml. *S. zooepidemicus* counts with linear and best fitting polynomial regression lines plotted. Graphs were plotted incorporating 3% spherical random noise around plotted points in order to avoid loss of information caused by overlaying of points.

**Figure 8.2: CDNS score vs. $log_{10}$ cfu/ml *S. zooepidemicus* in tracheal wash with data fitted from significant a) linear and b) best fitting polynomial regression models and including 3% spherical random noise. The deviance benefit between regression models was statistically significant (P<0.001)**

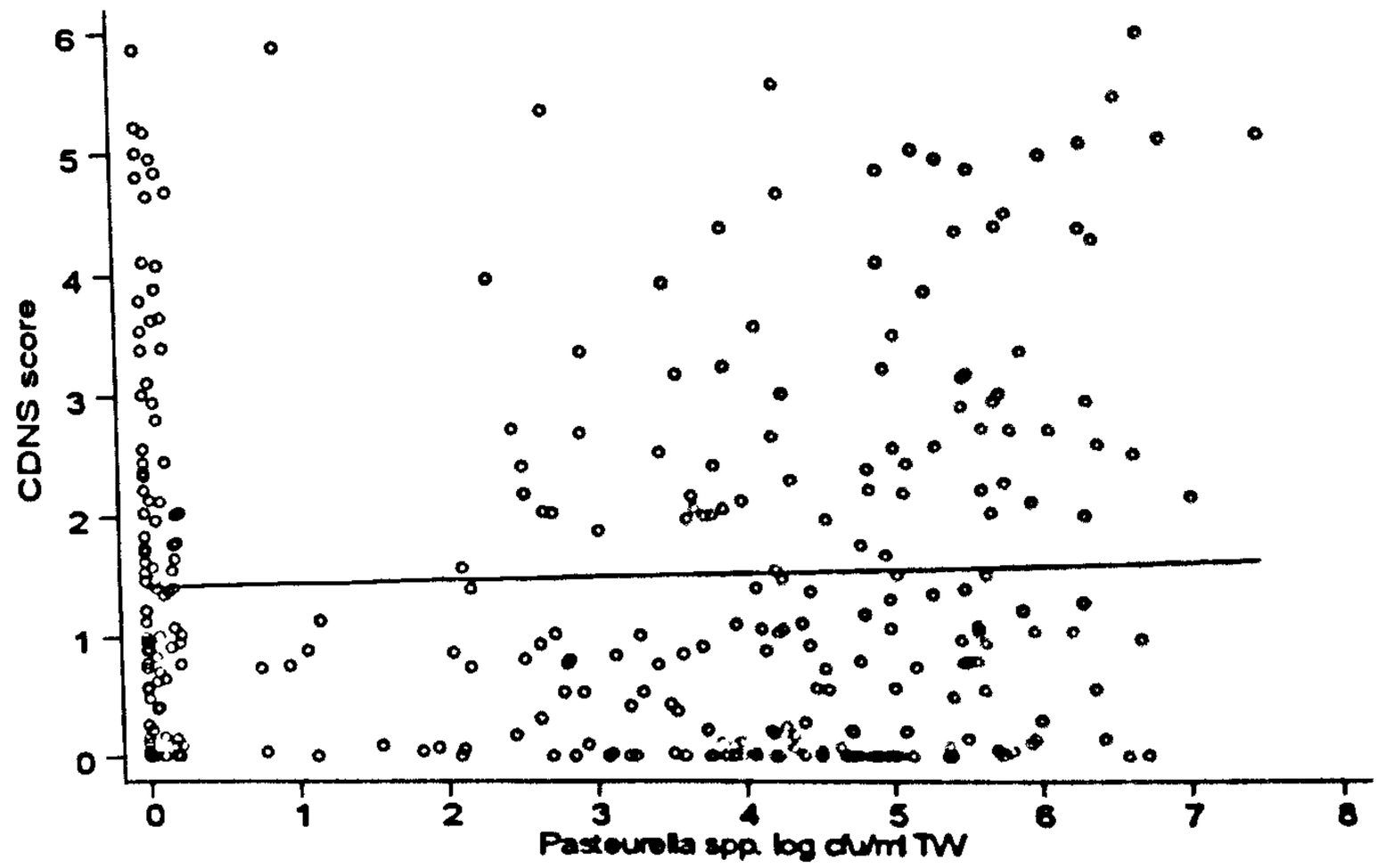



Results indicated that for $log_{10}$ cfu/ml *Pasteurella* spp. in tracheal washes there was no significant linear relationship with increasing clinical and CDNS scores but there was with airway inflammation score. The use of a best fitting, 2-power term polynomial regression model provided significant improvement over linear models for each of the 3 outcome measures. Figure 8.3 illustrates the distribution of CDNS scores versus corresponding $log_{10}$ cfu/ml. *Pasteurella* spp. counts with linear and best fitting polynomial

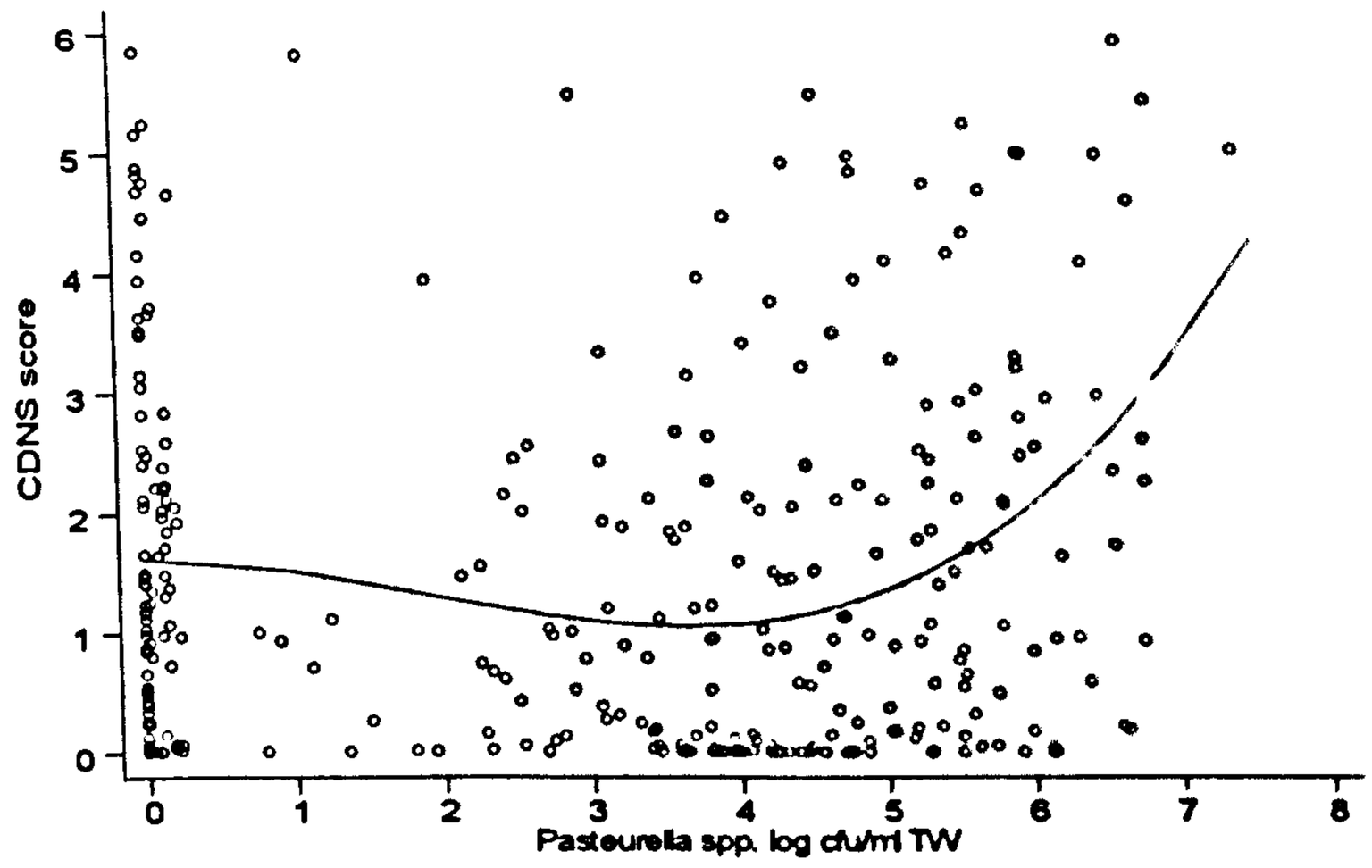
regression lines plotted. Graphs again incorporated 3% spherical random noise around plotted points.

**Figure 8.3: CDNS score vs. $log_{10}$ cfu/ml *Pasteurella* spp. in tracheal wash with data fitted from non-significant a) linear and significant b) best fitting polynomial regression models and including 3% spherical random noise. The deviance benefit between regression models was statistically significant (P<0.001)**

a)



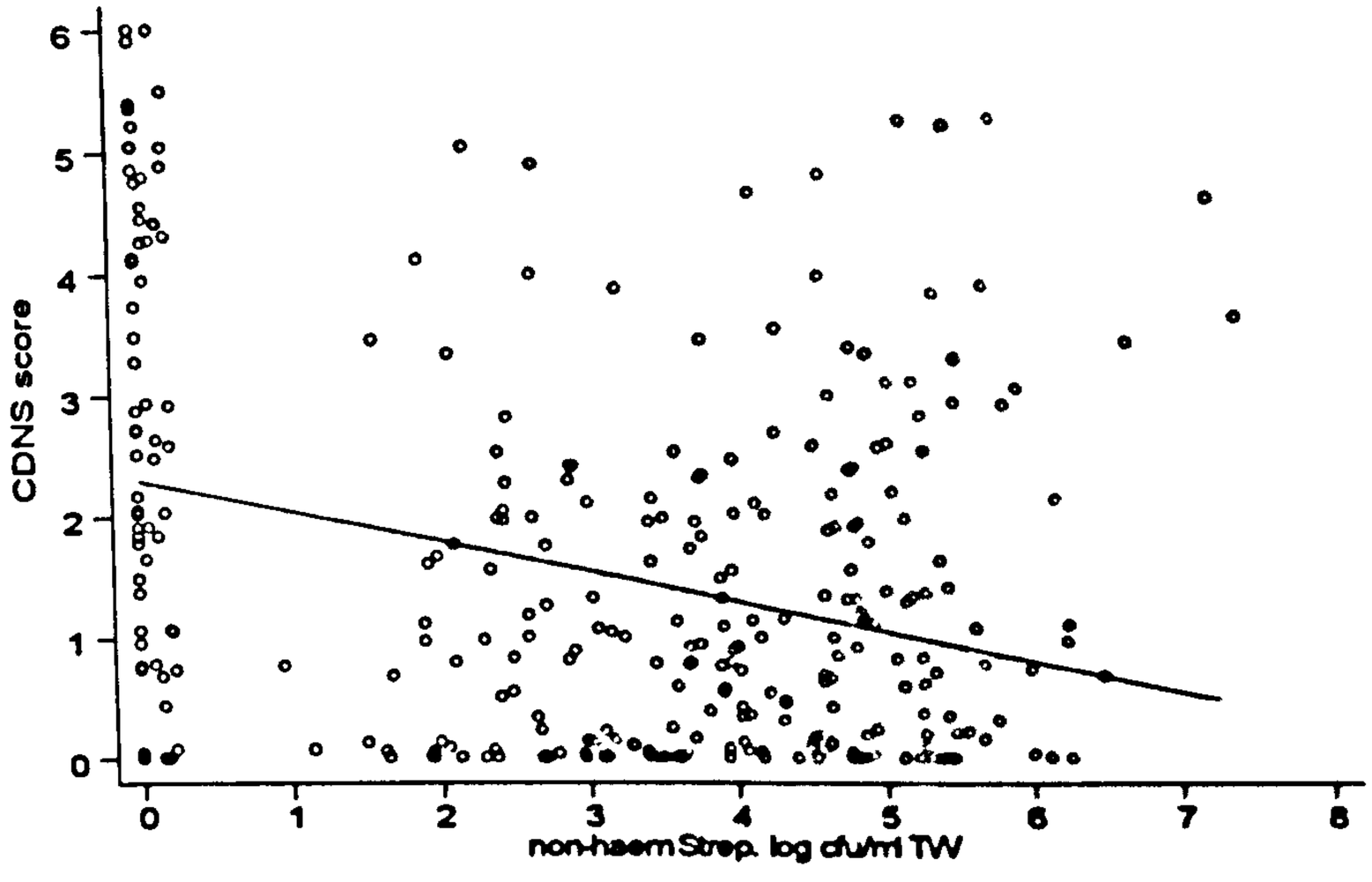

b)



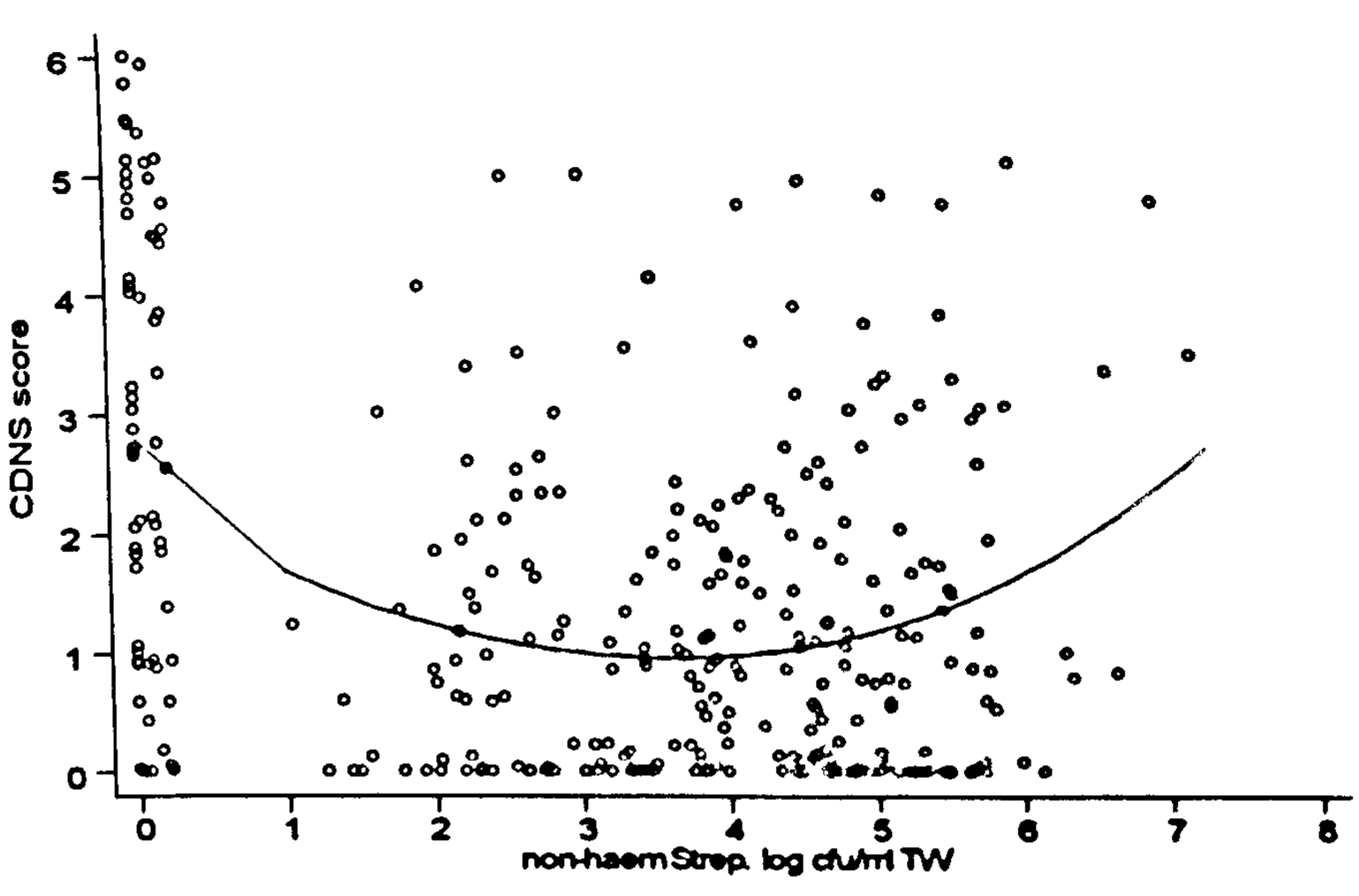
Results indicated that for $\log_{10}$ cfu/ml non-haemolytic *Streptococcus* spp. in tracheal washes there was a significant inverse linear relationship with increasing clinical and CDNS scores and a marginally significant inverse relationship with airway inflammation score. The use of a best fitting, 2-power term polynomial regression model provided significant improvement over linear models for each of the 3 outcome measures. Figure 8.4, incorporating 3% spherical random noise around plotted points, illustrates the distribution of CDNS scores versus corresponding $\log_{10}$ cfu/ml. non-haemolytic *Streptococcus* spp counts with linear and best fitting polynomial regression lines plotted.

**Figure 8.4: CDNS score vs. $\log_{10}$ cfu/ml non-haemolytic *Streptococcus* spp. in tracheal wash with data fitted from significant a) linear and b) best fitting polynomial regression models and including 3% spherical random noise. The deviance benefit between regression models was statistically significant (P<0.001)**

a)




b)

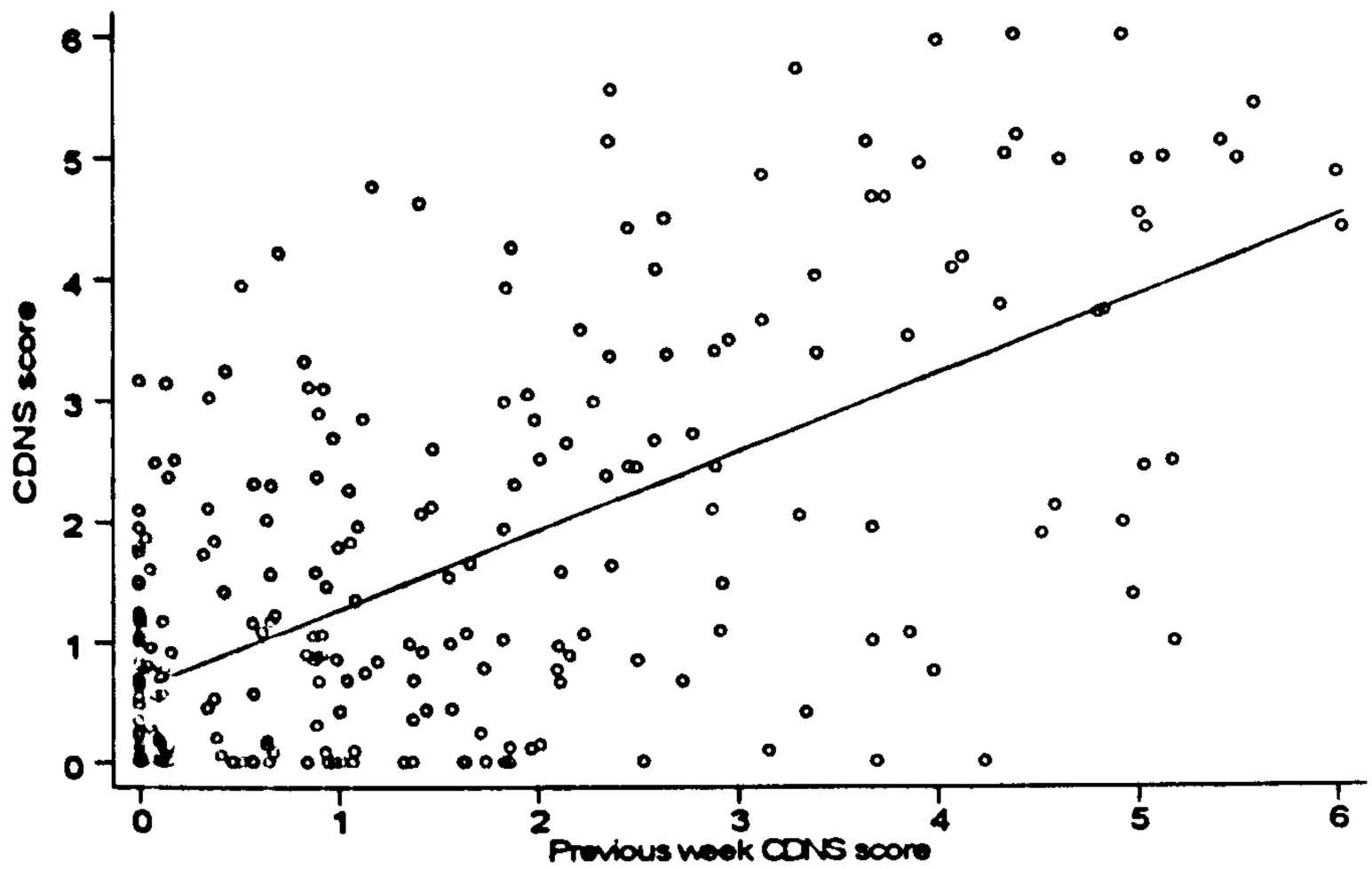



There was a significant linear relationship between increasing score for all 3 outcome measures and all levels of autoregressive variables. This confirmed, not surprisingly, that higher outcome scores were predicted for ponies that had higher outcome scores in the previous weeks of sampling. Use of a best fitting, 2-power term polynomial regression model only provided significant improvement over a linear model for the

autoregressive variables for CDNS score 3 weeks previously and airway inflammation score the previous week. Figures 8.5 and 8.6 illustrate the distribution of CDNS scores versus corresponding one week and 3 week previously autoregressive CDNS scores with linear and best fitting polynomial regression lines plotted.

**Figure 8.5: CDNS score vs. CDNS score the previous week with data fitted from significant a) linear and b) best fitting polynomial regression models and including 3% spherical random noise. The deviance benefit between regression models was not statistically significant (P=0.936)**

a)




b)

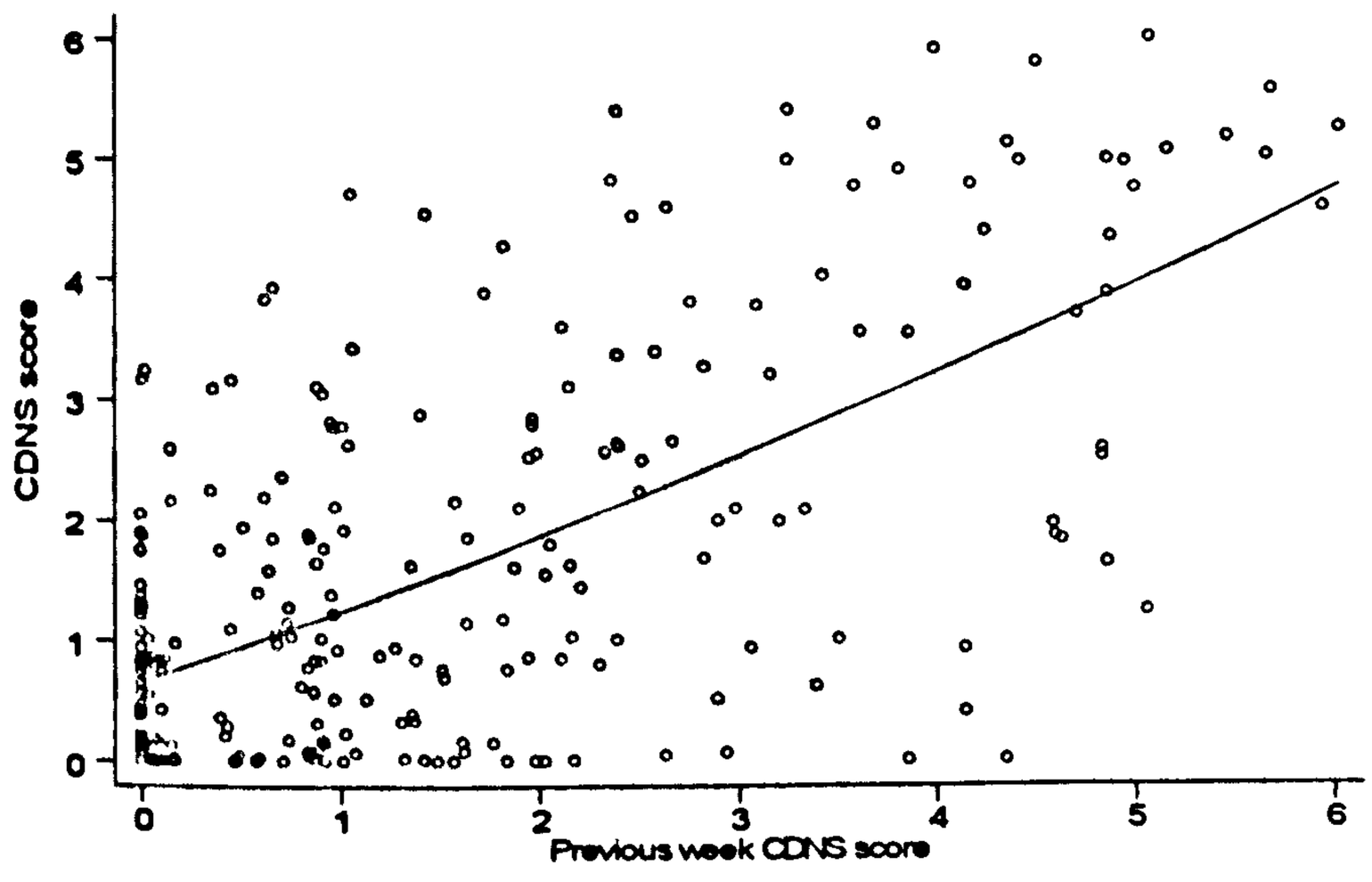
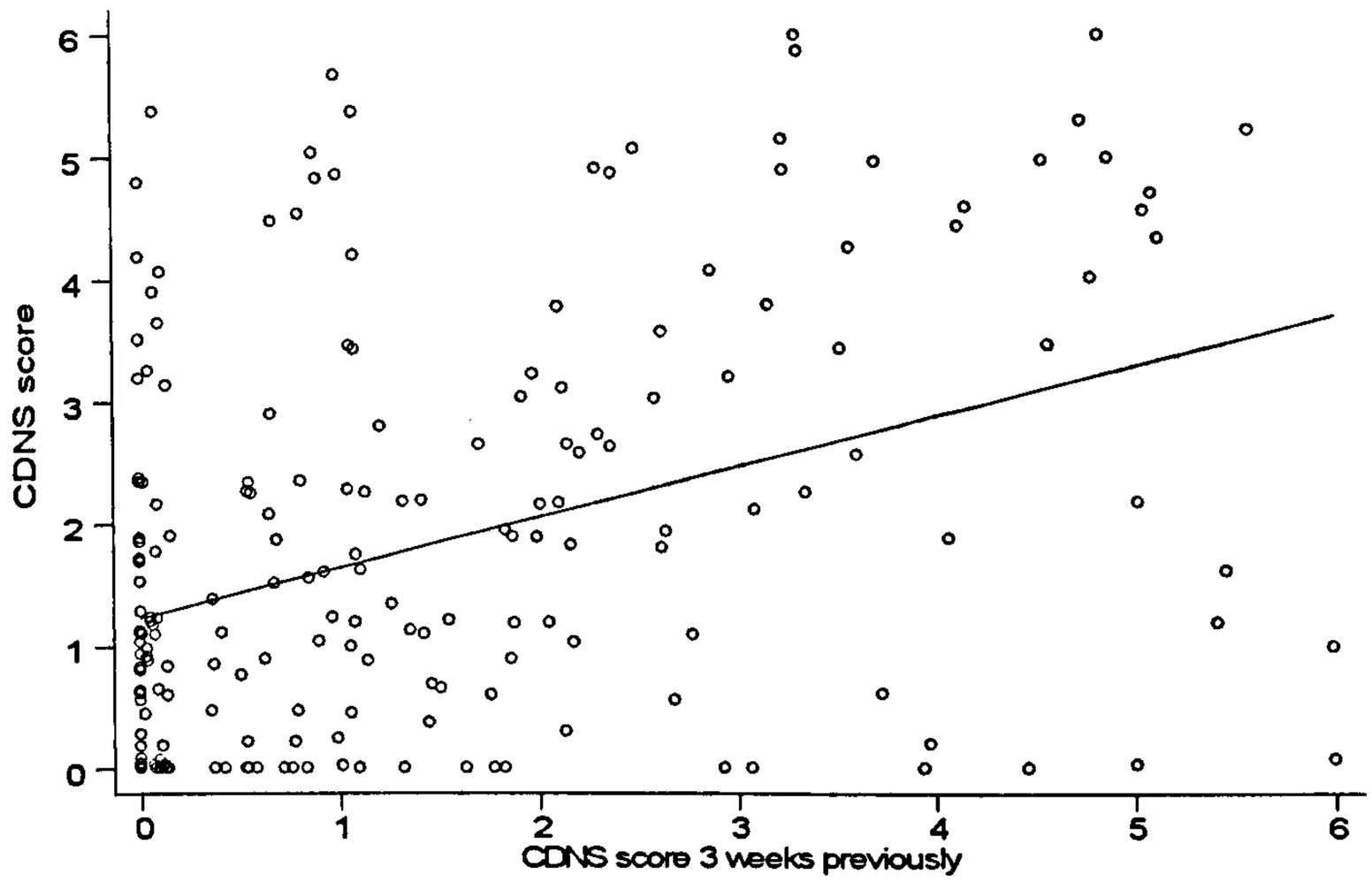
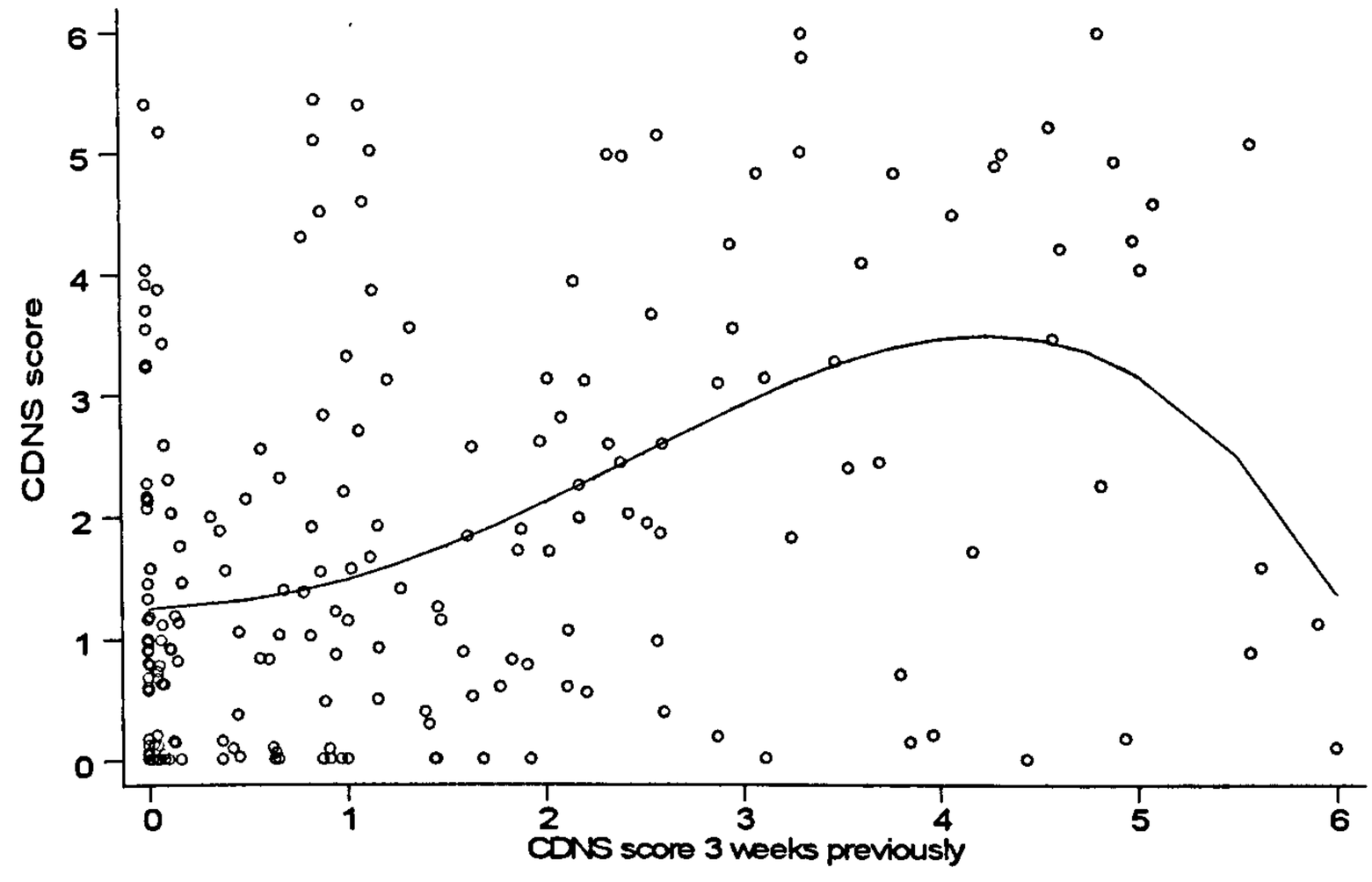


**Figure 8.6: CDNS score vs. CDNS score 3 weeks previously with data fitted from significant a) linear and b) best fitting polynomial regression models and including 3% spherical random noise. The deviance benefit between regression models was statistically significant (P=0.034)**

a)




b)




Examination of $R^2$ values demonstrated that the amount of variability in the outcomes that were explained by successive previous weeks' scores decreased over time. That is that the outcome score 3 weeks previously explained less variation in the current outcome score than the score 2 weeks previously, which in turn explained less than the score from the week immediately preceeding it.

Table A2.16 summarises the results of non-parametric tests to examine whether there were any significant differences in clinical and airway inflammation scores between the pony level variables of sex, vaccine group and each of the transferrin and protease inhibitor haplotypes. The observation level binary variables of isolation of different bacterial species from nasopharyngeal swabs were also examined.

Results indicated that for the aggregated clinical outcome measures there were statistically significant differences between the sexes, vaccine groups, transferrin D and F2 haplotypes, protease inhibitor L and L2 haplotypes and *S. zooepidemicus* isolates from nasopharyngeal swabs. Observations from female ponies and from those that did not receive vaccine or placebo but were introduced later, had significantly higher clinical score ranks. There was no significant difference between observations from vaccinates and controls when these observations were analysed alone (Wilcoxon rank sum test $P>0.28$). Observations from ponies either not possessing D or F2 transferrin haplotypes had significantly higher score ranks. Those without L or with L2 protease inhibitor haplotypes also had significantly higher clinical outcome ranks, although there were only observations from one and 3 ponies in these respective categories. Observations from ponies with protease inhibitor haplotype S had higher scores than those without. Those observations when *S. zooepidemicus* was isolated from the nasopharyngeal swab sample also had significantly higher clinical scores.

For airway inflammation score there were statistically significant differences between the sexes, transferrin F and H1 haplotypes, protease inhibitor L and L2 haplotypes and isolates of both *S. zooepidemicus* and *Pasteurella* spp. on nasopharyngeal swabs. For the appropriate variables the direction of the ranking was the same for airway inflammation score as it had been for the clinical outcomes. Observations when *Pasteurella* spp. was isolated from the nasopharyngeal swab sample and those from ponies without transferrin haplotype H1 both ranked significantly higher inflammation scores.

### *8.4.1.2 Ordinary multiple linear regression modelling*

Table 8.2 summarises the results from the final ordinary multiple linear regression models for the outcome variables of clinical score, CDNS score and airway inflammation score. Whilst all models included significant autoregressive variables there was no explicit controlling of within-pony relationships in these models. Two final models (Models 1 and 2) for both clinical and CDNS scores are presented. These represent respective models with transferrin D (Models 1) and transferrin F2 (Models 2) haplotypes retained as explanatory variables. Transferrin H1 was retained as a marginally significant in the final airway inflammation score model.

All final models were very similar with respect to the explanatory variables included in them. They all retained one or 2 autoregressive variables corresponding to the outcome parameter scores from the week and/or 2 weeks previously. This confirmed the importance of temporally related, repeated measure observations in these data. However, with inclusion of one or 2 autoregressive variables it was found that the variable corresponding to the outcome score from 3 weeks previously was not statistically significant in any model. Inclusion of autoregressive variables reduced the sample size from the original n=319 to n=229 for clinical score, to n=257 for CDNS score and to n=225 for airway inflammation score. All models also contained various terms for $\log_{10}$ cfu/ml counts of tracheal wash bacteria as significant explanatory factors and pony level factors relating to transferrin haplotypes were also retained. In the clinical score and CDNS score models, non-haemolytic *Streptococcus* spp. and *Pasteurella* spp. were both significant when expressed as quadratic terms and $\log_{10}$ cfu/ml *S. zooepidemicus* was retained as a significant linear term. Results for clinical and CDNS scores were consistent with those seen for the pony level analyses in that both transferrin D and F2 haplotypes were retained as potentially important predictive factors, particularly in the CDNS score model. In the airway inflammation score

model, non-haemolytic *Streptococcus* spp. as a quadratic term and *S. zooepidemicus* expressed as a linear term, were both significant and were retained.

**Table 8.2: Results of multivariable linear and polynomial regression of clinical, CDNS and airway inflammation parameter scores**

| Outcome variable / Model | Explanatory variables in the models (n) | Regression coefficient | 95% CI of coefficient | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|
| **Clinical score** | (n=229) | | | | |
| Model 1 | Intercept | 1.66 | 0.78 – 2.55 | 50.3 | <0.001 |
| | Clinical score 1 week previously | 0.41 | 0.28 – 0.54 | | <0.001 |
| | Clinical score 2 weeks previously | 0.14 | 0.02 – 0.27 | | 0.023 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.43 | -0.78 – -0.08 | | 0.016 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.07 | 0.01 – 0.12 | | 0.025 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.47 | -0.76 – -0.18 | | 0.002 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.08 | 0.03 – 0.13 | | 0.002 |
| | $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.15 | 0.02 – 0.27 | | 0.019 |
| | Transferrin D haplotype | -0.40 | -0.79 – -0.002 | | 0.051 |
| Model 2 | Intercept | 1.20 | 0.39 – 2.02 | 50.1 | 0.004 |
| | Clinical score 1 week previously | 0.42 | 0.29 – 0.55 | | <0.001 |
| | Clinical score 2 weeks previously | 0.14 | 0.01 – 0.26 | | 0.028 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.42 | -0.77 – -0.07 | | 0.019 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.07 | 0.01 – 0.13 | | 0.018 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.49 | -0.78 – -0.20 | | 0.001 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.08 | 0.03 – 0.13 | | 0.002 |
| | $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.13 | 0.01 – 0.26 | | 0.037 |
| | Transferrin F2 haplotype | 0.40 | -0.04 – 0.83 | | 0.075 |
| **CDNS score** | (n=257) | | | | |
| Model 1 | Intercept | 1.51 | 0.89 – 2.14 | 53.3 | <0.001 |
| | CDNS score 1 week previously | 0.49 | 0.38 – 0.59 | | <0.001 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.42 | -0.68 – -0.15 | | 0.002 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.05 | 0.01 – 0.10 | | 0.017 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.38 | -0.60 – -0.16 | | 0.001 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.07 | 0.03 – 0.10 | | 0.001 |
| | $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.11 | 0.01 – 0.20 | | 0.028 |
| | Transferrin D haplotype | -0.39 | -0.69 – -0.09 | | 0.010 |
| Model 2 | Intercept | 1.05 | 0.44 – 1.65 | 53.3 | 0.001 |
| | CDNS score 1 week previously | 0.49 | 0.38 – 0.59 | | <0.001 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.40 | -0.67 – -0.13 | | 0.004 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.06 | 0.01 – 0.10 | | 0.012 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.40 | -0.61 – -0.18 | | <0.001 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.07 | 0.03 – 0.10 | | 0.001 |
| | $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.10 | 0.0003 – 0.19 | | 0.049 |
| | Transferrin F2 haplotype | 0.41 | 0.10 – 0.73 | | 0.011 |
| **Airway inflammation (AI) score** | (n=225) | | | | |
| | Intercept | 3.06 | 2.03 – 4.09 | 41.8 | <0.001 |
| | AI score 1 week previously | 0.23 | 0.11 – 0.35 | | <0.001 |
| | AI score 2 weeks previously | 0.17 | 0.07 – 0.27 | | 0.001 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.37 | -0.71 – -0.03 | | 0.034 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.06 | 0.006 – 0.12 | | 0.030 |
| | $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.33 | 0.20 – 0.46 | | <0.001 |
| | Transferrin H1 haplotype | -0.63 | -1.24 – -0.02 | | 0.044 |

Examination of $R^2$ values for these models demonstrated that >50% of the variability in clinical and CDNS scores appeared to be explained by the factors retained in the final models. The corresponding figure for the airway inflammation score was around 42%.

### *8.4.1.3 Multilevel linear regression modelling*

Table 8.3 summarises the results from the multilevel linear and polynomial regression modelling process for the outcome variables of clinical score, CDNS score and airway inflammation score. Results represent the important stages of the model building process with the first models (Model 1) being those with significant autoregressive variables and a linear term for *S. zooepidemicus* included. There was no significant improvement with the use of a quadratic or polynomial term for *S. zooepidemicus* over the linear term. The autoregressive variables corresponding to outcomes 2 weeks previously were not significant for clinical and CDNS scores when included with the previous week autoregressive variable and the linear *S. zooepidemicus* term. Subsequent models illustrated results for the addition of other significant variables through to the final models (Models 4a and 4b for clinical and CDNS score and Model 3 for airway inflammation score).

Although it was possible that the random variability between ponies might have been due to differences in both their intercepts and their slopes (so called random intercept and slope models), multivariable modelling of these limited data showed that a random intercept only model was appropriate as their was no significant improvement by inclusion of random slope parameters. Also represented in the tables were the deviances (-2*loglikelihood) for each model. Where appropriate (same n), assessment of statistically significant improvement in the fit of models was made by calculation of the difference (reduction) in their deviances (likelihood ratio statistic LRS). The LRS followed a chi-squared distribution, with degrees of freedom equal to the difference in numbers of

parameters in models being compared and $P \leq 0.05$ was used as the criterion for significant improvement. Also represented for models were the intra-pony correlation, which was the proportion (%) of the total model variance attributable to variation between ponies. Therefore, the more similar ponies were to each other then the lower the intra-pony correlation.

**Table 8.3: Summary of multilevel linear and polynomial regression modelling of clinical, CDNS and airway inflammation scores**

| Outcome variable / Effect type / Explanatory variable in model | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) |
|---|---|---|---|---|---|
| **Clinical score** | **Model 1** n=257 | **Model 2** n=257 | **Model 3** n=257 | **Model 4a** n=257 | **Model 4b** n=257 |
| Fixed effect | | | | | |
| Intercept | 0.79 (0.25) | 1.52 (0.38) | 1.76 (0.38) | 2.20 (0.42) | 1.46 (0.39) |
| Clinical score 1 week previously | 0.35 (0.05) | 0.34 (0.05) | 0.36 (0.05) | 0.35 (0.05) | 0.35 (0.05) |
| $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.27 (0.05) | 0.21 (0.06) | 0.17 (0.06) | 0.16 (0.06) | 0.15 (0.06) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | | -0.43 (0.16) | -0.40 (0.16) | -0.40 (0.16) | -0.38 (0.16) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | | 0.06 (0.03) | 0.06 (0.03) | 0.06 (0.03) | 0.06 (0.03) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | | | -0.45 (0.13) | -0.44 (0.13) | -0.45 (0.13) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | | | 0.08 (0.02) | 0.08 (0.02) | 0.08 (0.02) |
| Transferrin D haplotype | | | | -0.63 (0.27) | |
| Tranfserrin F2 haplotype | | | | | 0.68 (0.27) |
| Random effect | | | | | |
| Level 2 variance: Pony | 0.48 (0.18) | 0.41 (0.16) | 0.36 (0.14) | 0.27 (0.12) | 0.27 (0.12) |
| Level 1 variance: Observation | 1.71 (0.16) | 1.68 (0.16) | 1.61 (0.15) | 1.61 (0.15) | 1.61 (0.15) |
| *-2*loglikelihood* | *903.20* | *896.09* | *883.88* | *878.49* | *877.80* |
| *LRS $\chi^2$ (d.f.)* | | *7.11 (2)* | *12.21 (2)* | *5.39 (1)* | *6.08 (1)* |
| *P-value* | | *0.029* | *0.002* | *0.020* | *0.014* |
| *Intra-pony correlation (%)* | *21.9* | *19.6* | *18.3* | *14.4* | *14.4* |
| **CDNS score** | **Model 1** n=257 | **Model 2** n=257 | **Model 3** n=257 | **Model 4a** n=257 | **Model 4b** n=257 |
| Fixed effect | | | | | |
| Intercept | 0.17 (0.20) | 1.02 (0.30) | 1.24 (0.30) | 1.56 (0.33) | 1.03 (0.31) |
| CDNS score 1 week previously | 0.43 (0.05) | 0.37 (0.05) | 0.39 (0.05) | 0.39 (0.05) | 0.39 (0.05) |
| $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.23 (0.05) | 0.18 (0.05) | 0.15 (0.05) | 0.14 (0.05) | 0.14 (0.05) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | | -0.38 (0.13) | -0.34 (0.13) | -0.35 (0.13) | -0.33 (0.13) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | | 0.04 (0.02) | 0.04 (0.02) | 0.04 (0.02) | 0.04 (0.02) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | | | -0.37 (0.11) | -0.36 (0.11) | -0.37 (0.11) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | | | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) |
| Transferrin D haplotype | | | | -0.46 (0.21) | |
| Transferrin F2 haplotype | | | | | 0.48 (0.21) |
| Random effect | | | | | |
| Level 2 variance: Pony | 0.26 (0.10) | 0.23 (0.10) | 0.20 (0.09) | 0.16 (0.07) | 0.16 (0.08) |
| Level 1 variance: Observation | 1.20 (0.11) | 1.14 (0.11) | 1.10 (0.10) | 1.10 (0.10) | 1.10 (0.10) |
| *-2*loglikelihood* | *806.58* | *792.96* | *781.38* | *776.70* | *776.50* |
| *LRS $\chi^2$ (1d.f.)* | | *13.62 (2)* | *11.58 (2)* | *4.68 (1)* | *4.88 (1)* |
| *P-value* | | *0.001* | *0.003* | *0.031* | *0.027* |
| *Intra-pony correlation (%)* | *17.8* | *16.8* | *15.4* | *12.7* | *12.7* |

Table 8.3 continued

| **Outcome variable** / Effect type / Explanatory variable in model | **Estimate (S.E.)** | **Estimate (S.E.)** | **Estimate (S.E.)** | **Estimate (S.E.)** | **Estimate (S.E.)** |
|---|---|---|---|---|---|
| **Airway inflammation (AI) score** | **Model 1** | **Model 2** | **Model 3** | | |
| | **n=225** | **n=225** | **n=225** | | |
| Fixed effect | | | | | |
| Intercept | 2.43 (0.39) | 2.88 (0.51) | 3.06 (0.52) | | |
| AI score 1 week previously | 0.24 (0.06) | 0.25 (0.06) | 0.23 (0.06) | | |
| AI score 2 weeks previously | 0.17 (0.05) | 0.17 (0.05) | 0.17 (0.05) | | |
| $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.39 (0.06) | 0.33 (0.06) | 0.33 (0.06) | | |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | | -0.36 (0.17) | -0.37 (0.17) | | |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | | 0.06 (0.03) | 0.06 (0.03) | | |
| Transferrin H1 haplotype | | | -0.63 (0.31) | | |
| Random effect | | | | | |
| Level 2 variance: Pony | 0.02 (0.08) | 0.02 (0.08) | 0.00 (0.00) | | |
| Level 1 variance: Observation | 1.98 (0.20) | 1.94 (0.20) | 1.94 (0.20) | | |
| *-2*loglikelihood* | *794.27* | *789.77* | *785.66* | | |
| *LRS $\chi^2$ (1d.f.)* | | *4.50 (2)* | *4.11 (1)* | | |
| *P-value* | | *0.105* | *0.043* | | |
| *Intra-pony correlation (%)* | *1.0* | *1.2* | *0.0* | | |

Sequential inclusion of quadratic terms for non-haemolytic *Streptococcus* spp. (Model 2) and *Pasteurella* spp. (Model 3) significantly improved the fit of models as measured by reduction of model deviance and likelihood ratio statistics. The values of the quadratic terms for these bacterial species demonstrated that there was a positive association only between outcome scores at the higher levels of bacterial counts (>$10^6$ $log_{10}$ cfu/ml) in tracheal washes but there was a negative association with low to moderate counts ($10^2$-$10^5$ $log_{10}$ cfu/ml). The final stage of model building for clinical and CDNS scores was the addition of variables for transferrin haplotypes. The separate inclusion of transferrin D (Models 4a) and F2 (Models 4b) haplotypes resulted in 2 largely equivalent final models for each of clinical and CDNS scores. Comparison of Models 4a and 4b with Model 3, which had the same sample size (n=257), showed that addition of the transferrin haplotype variables resulted in significant improvement in the fit of models as measured by reduction in deviance. This also resulted in a reduction in the pony level variance among the random effects and a consequent reduction in the intra-pony correlation. Therefore, inclusion of the pony-level variable for transferrin haplotype was explaining some of the difference between individual ponies that was being modelled as random variation. Inclusion of transferrin F2

haplotype resulted in a positive association, indicating that ponies with this haplotype had increased clinical and CDNS scores compared with those without this haplotype. Conversely, those ponies with the D haplotype had a negative association indicating that these ponies had lower outcome scores. For both the clinical and CDNS score models inclusion of transferrin F2 haplotype resulted in a marginal and non-significant improvement in model fit and reduction in deviance and intra-pony correlation compared with the model with transferrin D haplotype included.

In the multilevel linear regression modelling process for airway inflammation the autoregressive variable for outcome score 2 weeks previously remained statistically significantly associated with the outcome (Wald $\chi^2$, 1d.f. = 11.56; $P < 0.001$) with inclusion of other significant variables. In addition, there was no evidence for a significant pony-level random effect in these models. This was such that in the final model in which transferrin H1 haplotype was included, the level 2 variance was zero and the model was exactly equivalent to the final model produced from ordinary multiple linear regression analysis (Table 8.2).

Examination of pony level residuals

Using MLwiN software it was possible to examine the pony level residuals (n=29) of final fitted models and these were ranked and plotted with their 95% confidence intervals in so-called 'caterpillar' plots. Figures A2.7 and A2.8 represented caterpillar plots of the pony level residuals for the final models for clinical score including transferrin D and transferrin F2 haplotypes, respectively. Ponies with the largest residuals and with 95% confidence intervals that did not include or just included zero (hatched line representing zero residual is equivalent to the mean prediction from the fixed effects model) appeared at the extremes of the plots. Ponies 21 and 29 have been identified on Figure A2.7 as having the largest positive residuals, i.e. these ponies had higher clinical scores than was predicted by the final model including transferrin D haplotype. Also pony 28 was identified as the only

pony with a significantly negative residual value (95% confidence intervals for residual does not include zero), i.e. had lower clinical scores than was predicted by the final model. In the final model that included transferrin F2 haplotype, ponies 23 and 24 were identified as having the largest positive pony level residual and pony 28 again had the largest negative residual. Figures A2.9 and A2.10 represented caterpillar plots of the pony level residuals for the final models for CDNS score including transferrin D and transferrin F2 haplotypes, respectively. In these plots, there were no ponies in the transferrin D haplotype model with significantly positive residuals although pony 28 had a significant negative value residual in this model. In the transferrin F2 haplotype model pony 23 was identified as having the largest significant positive residual and pony 28 again had the residual with largest negative value.

Results of each stage in the further modelling of clinical score (Table A2.17) showed that as the ponies with largest value residuals were reassigned in the models there was a sequential decrease in deviance and the overall fit of models improved significantly at each stage. In all except one modelling stage (addition of pony 24 to Model 7b of clinical score with transferrin F2) there was an increase in the value (but in a negative direction for transferrin D) of the fixed effect slope estimates for the transferrin parameters. This was accompanied by a corresponding decrease in the standard errors for these estimates and hence the significance of their association with clinical score also increased. In addition, there was a notable decrease in both the estimates and significance of pony level variances across the sequential modelling, which also resulted in a sequential decrease in intra-pony correlation. The estimates and standard errors for the autoregressive and *S. zooepidemicus* fixed effect parameters and observation level variances remained largely unaltered during this modelling process.

Examination of the transferrin haplotype status of the ponies that had the largest value residuals revealed why these were likely to have had the largest influence on the

goodness of fit of the final clinical score models. In the model which included transferrin D haplotype, ponies 21 and 29 had the largest positive residuals, i.e. they had higher clinical scores than was predicted by the model. As these 2 ponies were each of the transferrin DF2 haplotype, they were consequently predicted by the model to have lower scores as they were positive for transferrin D (due to the negative fixed effect slope estimate for this parameter). In the case of pony 28, atypically for a transferrin F2 haplotype, the model predicted higher clinical scores than were actually observed for this individual which had a low clinical score and low *S. zooepidemicus* counts (Figure A2.6). Examination of the additional modelling of data for the clinical score model with transferrin F2 haplotype showed that pony 23 had the largest positive value residual and the greatest influence on model fit when reassigned in the model. Examination of data (Figure A2.6) showed that this was likely to be due to relatively low $\log_{10}$ cfu/ml *S. zooepidemicus* counts in tracheal washes compared to other ponies with comparatively high clinical scores. In this model the influence of pony 23 was exacerbated as this individual was also negative for transferrin F2 haplotype and hence the clinical scores observed were much higher than predicted by the model.

Table A2.18 summarises a similar further modelling of 2 final models for CDNS score, each including transferrin D (Model 4a) and transferrin F2 (Model 4b) haplotypes. For the transferrin D model, no ponies had significantly large positive value residuals and pony 28 had the only significant negative value residual, which was reassigned in re-modelling (Model 5a). In the transferrin F2 model, pony 23 had the largest positive and pony 28 the largest negative residual values. Results were very similar to those seen for the clinical score models with respect to decreasing pony level variances and intra-pony correlations, and, increasing estimate values and significances (due to decreasing standard error estimates) with exclusion of ponies with large value residuals from the random effect parts of models.

The final multilevel model (Model 3; Table 8.3) for airway inflammation had identical fixed effects (estimates and variables) to the multiple linear regression model (Table 8.2) and had zero pony-level variance. Consequently, this final model did not have pony-level residuals that varied from zero. The model that did not include the transferrin parameter (Model 2) did have a small amount of non-significant pony-level variance but all pony-level residuals for this model had 95% confidence intervals that did include zero (Figure A2.11).

### 8.4.2 Individual clinical signs

#### *8.4.2.1 Univariable analyses*

Tables A2.19a - e summarise the results of each univariable logistic regression analysis of individual clinical signs with explanatory variables, including the linear and categorical terms for counts of individual bacterial species in tracheal washes, autoregressive outcomes from previous weeks, sex, vaccine group, transferrin and protease inhibitor haplotypes and nasopharyngeal swab bacterial isolates.

Other than ocular discharge, which with the exceptions of *Pasteurella* spp. on swabs and $10^3$ - $10^5$ cfu/ml *B. bronchiseptica* in tracheal washes, did not show significant association with explanatory variables, the other clinical parameters were largely consistent in the variables with which they were significantly associated.

Nasal discharge, coughing, abnormal breathing/dyspnoea and SMLN enlargement were all significantly positively associated with increasing total tracheal wash bacterial counts and all measures (linear and categorical measures in tracheal washes and isolation from nasopharyngeal swab) of *S. zooepidemicus* infection. The same 4 signs were also significantly negatively associated with linear and categorical measures of non-haemolytic *Streptococcus* spp. in tracheal washes. Ponies with *Pasteurella* spp. $>10^6$ cfu/ml in tracheal washes had significantly increased risk of abnormal breathing/dyspnoea (OR 2.7, P = 0.026)

and SMLN enlargement (OR 2.6, P = 0.036), compared with ponies with $<10^4$ cfu/ml. There was no evidence of a significant association of *Pasteurella* spp. with these signs at counts in tracheal washes between $10^4$ and $10^6$ cfu/ml.

The majority of autoregressive outcome variables were highly statistically significantly associated (P<0.001) with the 4 clinical parameters of nasal discharge, coughing, abnormal breathing/dyspnoea and SMLN enlargement. With the exceptions of nasal discharge 3 weeks previously and abnormal breathing/dyspnoea 2 weeks previously that had the highest risk associated with them, there was a general trend towards reducing risk of demonstrating a clinical sign with increasing time period between previous week and time of observation. This was illustrated by coughing. Ponies that had coughed the previous week were 6.2 times more likely to cough in the current week whereas ponies that had coughed 2 and 3 weeks previously were 4.3 times and 2.9 times respectively, more likely to cough.

For nasal discharge, coughing and abnormal breathing/dyspnoea there was a significant negative association between clinical outcome and being male and possession of transferrin D haplotype. In contrast, there was a significant positive association between these 3 outcomes and possession of transferrin F2 haplotype.

The categorical variable of vaccine group was significantly positively associated with coughing and SMLN enlargement in these univariable analyses. Ponies that received placebo were at approximately 3 times the risk of coughing than those ponies that were vaccinated and the late introduction animals were at almost 5 times the risk of SMLN enlargement compared to both vaccinates or controls.

There were other occasionally significant associations, particularly involving transferrin H1 haplotype, and protease inhibitor L and L2 haplotypes. Examination of the frequency of these haplotypes in the dataset (Table 7.2), showed that they occurred or were absent in relatively very few animals and their association with disease was consequently

strongly influenced by the frequent presence or absence of signs in certain of these individuals.

An example of this is the significant association between possession of transferrin H1 haplotype and coughing (OR 3.3, P = 0.004). Examination of the frequency of transferrin haplotypes in Table 7.2 shows that there were only 3 ponies with transferrin H1 haplotype. One of these, pony 24, had the highest cumulative cough score (Table A2.4) indicating that of all the ponies it suffered the most coughing over the study period. Results of a logistic regression analysis for coughing with all results from pony 24 excluded showed that there was no longer any association between coughing and transferrin H1 haplotype (OR 1.0, P = 0.963). This confirms that extreme caution is required in interpreting the findings of these analyses where small numbers of individuals comprise categories of explanatory variables.

Logistic regression analysis was unable to fit models (i.e. there was non-convergence) for the association of isolation of *Pasteurella*-like spp. from nasopharyngeal swab extracts with ocular discharge, coughing or abnormal breathing/dyspnoea. This was because in all of these outcomes there were no cases (i.e. ponies demonstrating the clinical sign in question) from which *Pasteurella*-like spp. were not isolated and therefore the presence of these bacteria on swabs predicted each outcome perfectly. In order to allow examination of the effect of *Pasteurella*-like spp. isolated from nasopharyngeal swabs in univariable and later multivariable analyses, data were slightly modified to create a single, artificial negative nasopharyngeal swab isolation of *Pasteurella*-like spp. from a clinical case. The nasopharyngeal *Pasteurella*-like spp. culture result for pony 14 on week 5 was modified (i.e. binary classification of one changed to zero). This pony at this time was positive for ocular discharge, coughing and abnormal breathing/dyspnoea and being an observation from week 5 (rather than weeks one, 2, 3 or 26) would allow its inclusion with all levels of autoregressive variables. Results of univariable analyses using the modified data

showed that isolation of *Pasteurella*-like spp. from the nasopharynx was significantly associated with both ocular discharge and abnormal breathing/dyspnoea but was not significantly associated with coughing.

### *8.4.2.2 Multivariable analyses*

Table 8.4 summarises the final multivariable logistic regression models that include pony-level random effects for each of the individual clinical outcomes of nasal discharge, coughing, abnormal breathing/dyspnoea and SMLN enlargement. Results of multivariable logistic regression analyses incorporating pony-level random effects are now discussed for each of the individual clinical signs.

<u>Ocular discharge</u>

Multivariable logistic regression modelling of ocular discharge resulted in no significant improvement of association of any explanatory variable with this outcome, therefore results are not presented. This demonstrated that ocular discharge was not well predicted by any of the factors examined in this study, including autoregressive variables. The results of univariable and multivariable logistic regression analyses were consistent with ocular discharge not being part of the complex of clinical signs normally associated with clinical equine respiratory disease. The lack of association between ocular discharge and the majority of risk factors examined was in contrast to other signs. In keeping with the absence of significant results in earlier pony-level analyses for this sign, this suggested strongly that inclusion of ocular discharge was not warranted or necessary in a summary measure of respiratory disease. This was consistent with the summary measure of clinical score (including ocular discharge) compared to CDNS score (excluding ocular discharge) showing no difference in the variables with which it was significantly associated but

possessing more variability or 'noise', as evidenced by decreased $R^2$ values in both univariable and multivariable analyses.

**Table 8.4: Summary of comparisons of 3 different estimation methods for final multivariable logistic regression models including pony-level random effects for individual clinical outcomes (except ocular discharge)**

| Clinical outcome being modelled (n) | MLwiN 2nd order PQL RIGLS* estimates | | MLwiN 2nd order PQL IGLS** estimates | | Egret software maximum likelihood estimates (MLE) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Explanatory variables in model | β | S.E. β | β | S.E. β | β | S.E. β | Odds ratio | 95% CI | P-value |
| **Nasal discharge (n=202)** | | | | | | | | | |
| Intercept | -0.84 | 0.51 | -0.95 | 0.47 | -0.91 | 0.54 | | | |
| Nasal discharge 1 week previously | -0.44 | 0.41 | -0.32 | 0.39 | -0.37 | 0.49 | 0.69 | 0.26 – 1.80 | 0.450 |
| Nasal discharge 2 weeks previously | -0.40 | 0.41 | -0.30 | 0.40 | -0.33 | 0.47 | 0.72 | 0.29 – 1.78 | 0.474 |
| Nasal discharge 3 weeks previously | 1.04 | 0.42 | 1.07 | 0.41 | 1.05 | 0.42 | 2.87 | 1.26 – 6.54 | 0.012 |
| $10^4$-$10^5$ cfu/ml TW *S. zooepidemicus* | 0.47 | 0.52 | 0.47 | 0.50 | 0.47 | 0.52 | 1.60 | 0.58 – 4.44 | 0.364 |
| $10^5$-$10^6$ cfu/ml TW *S. zooepidemicus* | 2.89 | 0.96 | 2.83 | 0.92 | 2.83 | 0.89 | 17.0 | 2.94 – 98.3 | 0.002 |
| >$10^6$ cfu/ml TW *S. zooepidemicus* | 4.12 | 1.07 | 3.87 | 1.00 | 3.95 | 1.13 | 51.8 | 5.66 – 474.7 | <0.001 |
| NP *B. bronchiseptica* | 1.08 | 0.49 | 1.08 | 0.47 | 1.09 | 0.48 | 2.97 | 1.15 – 7.65 | 0.024 |
| Pony level variance | 1.51 | 0.64 | 1.09 | 0.52 | 1.21 | 0.92 | | | |
| **Coughing (n=257)** | | | | | | | | | |
| *Model 1* | | | | | | | | | |
| Intercept | -2.80 | 0.73 | -2.66 | 0.61 | -2.66 | 0.61 | | | |
| Coughing 1 week previously | 0.75 | 0.47 | 0.94 | 0.45 | 0.94 | 0.50 | 2.57 | 0.96 – 6.91 | 0.062 |
| Vaccine group: placebo | 1.67 | 0.67 | 1.52 | 0.55 | 1.51 | 0.56 | 4.53 | 1.52 – 13.5 | 0.007 |
| Vaccine group: late introduction | -0.52 | 0.93 | -0.50 | 0.77 | -0.51 | 0.76 | 0.60 | 0.14 – 2.67 | 0.505 |
| $10^4$-$10^5$ cfu/ml TW *S. zooepidemicus* | 0.55 | 0.59 | 0.51 | 0.56 | 0.51 | 0.56 | 1.66 | 0.55 – 4.98 | 0.365 |
| $10^5$-$10^6$ cfu/ml TW *S. zooepidemicus* | 1.48 | 0.66 | 1.45 | 0.61 | 1.45 | 0.61 | 4.25 | 1.29 – 14.0 | 0.018 |
| >$10^6$ cfu/ml TW *S. zooepidemicus* | 2.82 | 0.65 | 2.70 | 0.61 | 2.70 | 0.61 | 14.9 | 4.47 – 49.6 | <0.001 |
| Transferrin D | -1.08 | 0.61 | -0.99 | 0.49 | -0.98 | 0.49 | 0.38 | 0.14 – 0.99 | 0.047 |
| Pony level variance | 0.88 | 0.55 | 0.36 | 0.36 | 0.35 | 0.45 | | | |
| *Model 2* | | | | | | | | | |
| Intercept | -4.40 | 0.82 | -4.12 | 0.69 | -4.13 | 0.73 | | | |
| Coughing 1 week previously | 0.67 | 0.47 | 0.83 | 0.45 | 0.82 | 0.50 | 2.27 | 0.85 – 6.09 | 0.104 |
| Vaccine group: placebo | 2.11 | 0.74 | 1.95 | 0.62 | 1.97 | 0.64 | 7.14 | 2.04 – 25.0 | 0.002 |
| Vaccine group: late introduction | -0.48 | 0.93 | -0.41 | 0.77 | -0.41 | 0.77 | 0.66 | 0.15 – 3.01 | 0.595 |
| $10^4$-$10^5$ cfu/ml TW *S. zooepidemicus* | 0.59 | 0.59 | 0.57 | 0.57 | 0.58 | 0.56 | 1.78 | 0.59 – 5.33 | 0.304 |
| $10^5$-$10^6$ cfu/ml TW *S. zooepidemicus* | 1.32 | 0.67 | 1.27 | 0.63 | 1.27 | 0.63 | 3.55 | 1.03 – 12.3 | 0.046 |
| >$10^6$ cfu/ml TW *S. zooepidemicus* | 2.65 | 0.66 | 2.52 | 0.62 | 2.51 | 0.63 | 12.3 | 3.58 – 42.4 | <0.001 |
| Transferrin F2 | 1.46 | 0.70 | 1.30 | 0.58 | 1.31 | 0.60 | 3.69 | 1.13 – 12.1 | 0.031 |
| Pony level variance | 0.95 | 0.58 | 0.45 | 0.39 | 0.46 | 0.48 | | | |

*PQL RIGLS = Penalised QuasiLikelihood Restricted Iterative Generalised Least Squares **PQL IGLS = Penalised QuasiLikelihood Iterative Generalised Least Squares

Table 8.4 continued

| Clinical outcome being modelled (n) | MLwiN 2nd order PQL RIGLS* estimates | | MLwiN 2nd order PQL IGLS** estimates | | Egret software maximum likelihood estimates (MLE) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Explanatory variables in model | β | S.E. β | β | S.E. β | β | S.E. β | Odds ratio | 95% CI | P-value |
| **Abnormal breathing/dyspnoea (n=229)** | | | | | | | | | |
| *Model 1* | | | | | | | | | |
| Intercept | -1.04 | 0.59 | -1.14 | 0.54 | -1.10 | 0.59 | | | |
| Dyspnoea 1 week previously | 0.21 | 0.38 | 0.33 | 0.37 | 0.32 | 0.45 | 1.38 | 0.57 – 3.32 | 0.479 |
| Dyspnoea 2 weeks previously | 0.77 | 0.38 | 0.85 | 0.37 | 0.84 | 0.41 | 2.31 | 1.04 – 5.15 | 0.041 |
| TW $\log_{10}$ cfu/ml TW *S. zooepidemicus* | 0.26 | 0.11 | 0.25 | 0.10 | 0.25 | 0.10 | 1.28 | 1.04 – 1.57 | 0.017 |
| Transferrin D | -1.63 | 0.53 | -1.50 | 0.47 | -1.53 | 0.55 | 0.22 | 0.07 – 0.63 | 0.005 |
| Pony level variance | 0.99 | 0.50 | 0.64 | 0.39 | 0.67 | 0.61 | | | |
| *Model 2* | | | | | | | | | |
| Intercept | -2.72 | 0.54 | -2.65 | 0.51 | -2.64 | 0.52 | | | |
| Dyspnoea 1 week previously | 0.14 | 0.39 | 0.24 | 0.38 | 0.24 | 0.44 | 1.26 | 0.53 – 3.01 | 0.596 |
| Dyspnoea 2 weeks previously | 0.68 | 0.38 | 0.74 | 0.37 | 0.73 | 0.41 | 2.09 | 0.95 – 4.62 | 0.068 |
| TW $\log_{10}$ cfu/ml TW *S. zooepidemicus* | 0.20 | 0.11 | 0.19 | 0.11 | 0.19 | 0.11 | 1.21 | 0.98 – 1.50 | 0.076 |
| Transferrin F2 | 1.84 | 0.55 | 1.72 | 0.50 | 1.73 | 0.58 | 5.66 | 1.81 – 17.7 | 0.003 |
| Pony level variance | 1.01 | 0.50 | 0.72 | 0.42 | 0.74 | 0.60 | | | |
| **SMLN enlargement (n=261)** | | | | | | | | | |
| Intercept | -1.94 | 0.50 | -1.89 | 0.44 | -1.91 | 0.46 | | | |
| SMLN enlargement 1 week previously | 1.47 | 0.37 | 1.55 | 0.37 | 1.51 | 0.41 | 4.51 | 2.02 – 10.1 | <0.001 |
| Vaccine group: placebo | 0.30 | 0.67 | 0.27 | 0.59 | 0.31 | 0.60 | 1.36 | 0.42 – 4.44 | 0.608 |
| Vaccine group: late introduction | 2.05 | 0.87 | 1.92 | 0.76 | 2.01 | 0.82 | 7.44 | 1.49 – 37.1 | 0.014 |
| Pony level variance | 1.61 | 0.68 | 1.13 | 0.53 | 1.19 | 0.76 | | | |

*PQL RIGLS = Penalised QuasiLikelihood Restricted Iterative Generalised Least Squares **PQL IGLS = Penalised QuasiLikelihood Iterative Generalised Least Squares

Nasal discharge

The final logistic regression model for nasal discharge included all 3 autoregressive variables, terms for a categorical classification of *S. zooepidemicus* isolated in tracheal washes, a binary term for the isolation of *Bordetella bronchiseptica* from the nasopharynx and a pony-level random effect term.

Examination of the final model showed that among the autoregressive variables, a nasal discharge 3 weeks previously was significantly associated with increased risk of the same sign in any given week (adjusted OR 2.9, P = 0.012). It was possible that there might have been correlation between clinical signs and outcomes during any of the 3 weeks immediately preceeding it. Therefore, where there was a significant autoregressive variable corresponding to 2 or 3 weeks previously (thus reducing the effective sample size), the autoregressive variable(s) for the closer weeks would be retained *a priori*, irrespective of whether they themselves were significantly associated with the clinical outcome. This would not further affect the sample size in these models but would best allow for control of potential confounding effects from correlated outcomes.

Examination graphically of how the risk of nasal discharge changed with increasing $\log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes (Figure A2.12a) revealed there was not a satisfactory linear relationship between the logit of disease risk and $\log_{10}$ cfu/ml *S. zooepidemicus*. In order to represent most accurately the plateau of non-increased risk up to $10^4$ cfu/ml followed by a steep rise in risk with increasing *S. zooepidemicus* counts, a categorical representation of $\log_{10}$ cfu/ml *S. zooepidemicus* was used. The baseline group corresponded to washes with $<10^4$ cfu/ml with further categories for $10^4$-$10^5$ cfu/ml, $10^5$-$10^6$ cfu/ml and $>10^6$ cfu/ml. Inclusion of this categorical variable showed that increasing numbers of *S. zooepidemicus* in tracheal washes was associated with a significant increased risk of nasal discharge. There was some evidence that this increase in risk was dose dependent above $10^5$ cfu/ml.

Controlling for the other variables in the final model, isolation of *Bordetella bronchiseptica* was found to be associated with an increased risk of nasal discharge (adjusted OR 3.0, P = 0.024), even though it had not been statistically significant in univariable analysis (unadjusted OR 1.4, P = 0.188). Similarly, terms for non-haemolytic *Streptococcus* spp. in tracheal washes were no longer significantly associated with nasal discharge after controlling for other variables.

As these data were highly correlated within individual ponies all final models were produced *a priori* including a pony-level random effects term in order to account for random variability between ponies. Due to the lack of approximation to a chi-squared distribution of differences in deviances between models with and without a random effect terms and consequent difficulty in their interpretation, formal likelihood ratio statistics and corresponding P-values were not presented. However, there was evidence from the relatively larger coefficient estimate compared to its standard error (i.e. $\beta$: S.E.$\beta$ ratio $\geq 2.1$) that for the nasal discharge model the random effect term was likely to be statistically significant and its inclusion had improved the fit of the model.

As described in the methods, 3 different estimation techniques were used in 2 different software packages and their results compared. Table 8.4 provides the beta coefficient estimates and corresponding standard errors for restricted and non-restricted iterative generalised least squares (RIGLS and IGLS) algorithms using second order penalised quasilikelihood (PQL) estimation in MLwiN and maximum likelihood estimation (MLE) using a modified Newton-Raphson algorithm in Egret. The pony-level variance estimates and standard errors presented in Table 8.4 for the ML estimates from Egret have been modified so as to be consistent with those produced by MLwiN. The modifications were performed according to the relationship between different outputs as described by Snijders & Bosker (1999). The estimated adjusted odds ratios (OR) with 95% confidence

intervals around the estimates and corresponding Wald $\chi^2$ P-values produced by Egret are also presented.

Generally for all models there was very good agreement in the coefficient estimates and their standard errors for each of the 3 estimation techniques, with the second order PQL IGLS estimates and the maximum likelihood estimates producing almost identical results for most model components. No statistically significant interaction terms were identified.

Using MLwiN software it was possible to examine the pony level residuals (n=29) of final fitted logistic regression models with random effects and these were ranked and plotted with their 95% confidence intervals in 'caterpillar' plots. Figures A2.13 and A2.14 represented caterpillar plots of the pony level residuals for the final models for nasal discharge calculated by IGLS and RIGLS algorithms, respectively. It can be seen that the plots were virtually identical and that although all RIGLS estimated residuals were in the same direction (i.e. positive or negative) as the corresponding IGLS estimates, they were of consistently greater magnitude. Examination of the pony level residuals demonstrated that residuals for 2 ponies (ponies 5 and 28) had 95% confidence intervals that did not include zero.

Ponies with the largest residuals and with 95% confidence intervals that did not include or just included zero (hatched line representing zero residual is equivalent to perfect prediction by the model) appear at the extremes of the plots. Ponies 5 and 25 have been identified on Figures A2.13 and A2.14 as having the largest positive residuals, i.e. these ponies had more nasal discharges than were predicted by the final model. Also pony 28 was identified as the only pony with a significantly (95% confidence intervals for residual does not include zero) negative residual value, i.e. this pony had less nasal discharge than the final model predicted.

Examination of data (Figure A2.6) from ponies 5 and 25 that had the largest positive value residuals revealed why these animals were likely to have had an influence on the goodness of fit of the nasal discharge model. These ponies for several of the study weeks had nasal discharges but had low $\log_{10}$ cfu/ml *S. zooepidemicus*, lacked positive autoregressive outcomes 3 weeks previously and did not have *B. bronchiseptica* isolated from nasopharyngeal swabs. Examination of data (Figure A2.6) from pony 28 that had the largest negative value residual, revealed that for several of the study weeks this animal had no nasal discharge but had high $\log_{10}$ cfu/ml *S. zooepidemicus*, had positive autoregressive outcomes 3 weeks previously and had *B. bronchiseptica* isolated from nasopharyngeal swabs.

Coughing

As with the results of multivariable analyses of aggregated clinical outcome scores, 2 final models, corresponding to inclusion of either transferrin D (Model 1) or F2 (Model 2) haplotypes, were derived for the logistic regression analysis of the binary clinical outcome of coughing. Other significant or marginally significant and/or *a priori* variables in the final coughing models included the autoregressive variable of coughing the previous week (marginal), vaccine group category, the categorical classification of *S. zooepidemicus* in tracheal washes and pony-level random effect term (*a priori*).

In order to control for some of the temporal association between repeated weekly sampling and subsequent observation of the clinical sign, the autoregressive variable of coughing the previous week was retained in both models. Although only marginally statistically significant, there was evidence for a greater than 2-fold increase in risk of coughing when coughing had been present the previous week.

The categorical variable of vaccine group was retained as a statistically significant variable in both final coughing models. Results showed that there was an increased risk of

coughing among ponies that had received placebo compared to those that had been vaccinated but there was no statistically significant difference in risk of coughing between vaccinates and the 5 non-vaccinated ponies that were introduced later.

Examination graphically of how the risk of coughing changed with increasing $\log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes (Figure A2.12b) showed there was not a satisfactory linear relationship between the logit of disease risk and $\log_{10}$ cfu/ml *S. zooepidemicus*. However, the same categorical classification of $\log_{10}$ cfu/ml *S. zooepidemicus* used for nasal discharge (4 categories) was considered appropriate to represent the non-linearity of the logit of risk of coughing with increasing bacterial counts. Inclusion of this categorical variable showed that increasing numbers of *S. zooepidemicus* in tracheal washes was associated with a significant increased risk of coughing and again there was some evidence that this increase in risk was dose dependent above $10^5$ cfu/ml.

Binary variables for possession of either of 2 transferrin haplotypes (type D in Model 1 and type F2 in Model 2) remained significantly associated with coughing in these final models. The direction of the effect of each of these variables was the same as had been observed in previous analyses of aggregated clinical outcome scores. That is that possession of the transferrin D haplotype was associated with reduced risk of coughing (adjusted OR 0.4, P = 0.047) whereas possession of the F2 haplotype was associated with significantly increased risk (adjusted OR 3.7, P = 0.031).

Examination of the pony-level variance estimate and its standard error showed that these variables were unlikely to be statistically significant (i.e. $\beta$: S.E.$\beta$ ratio $\leq 1.94$) in the 2 coughing models. However, this variable was retained *a priori* to account for the hierarchical structure of repeated measures data within ponies in the dataset. No statistically significant interaction terms were identified.

Figures A2.15 and A2.16 represent caterpillar plots of the pony level residuals for the final coughing models calculated by RIGLS algorithms, which include transferrin D and F2 haplotypes, respectively.

Residuals for IGLS estimates (not shown), as for those demonstrated previously for nasal discharge, were more conservative in that they had smaller absolute values and the 95% confidence intervals for all pony level residuals included zero.

Examination of the RIGLS pony level residuals and their 95% confidence intervals for the final coughing model including transferrin D haplotype showed that the confidence intervals for only one pony (pony 12) did not include zero. The residual for pony 12 had a positive value indicating that this animal coughed on more occasions than was predicted by the model. Examination of data (Figure A2.6) revealed that pony 12, which as well as being in the vaccinated group and being positive for transferrin D haplotype (by virtue of being DF2 phenotype) also lacked positive autocorrelated outcomes from the previous week on 3 occasions.

Examination of the RIGLS pony level residuals and their 95% confidence intervals for the final coughing model that included transferrin F2 haplotype showed that the confidence intervals for only one pony (pony 23) did not include zero. The residual for pony 23 also had a positive value indicating that this animal coughed on more occasions than was predicted by the model. Examination of data (Figure A2.6) revealed that pony 23, which, despite being in the placebo group, was negative for the F2 haplotype (by virtue of being OH2 phenotype), lacked positive autocorrelated outcomes from the previous week on 2 occasions and had relatively low $\log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes.

Abnormal breathing/dyspnoea

As with the results for coughing, 2 final models that included different transferrin haplotypes (type D in Model 1 and type F2 in Model 2) were produced for the logistic

regression analysis of the binary outcome variable of abnormal breathing/dyspnoea. Other variables included in these models were autoregressive variables for abnormal breathing/dyspnoea one week and 2 weeks previously, $log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes and pony-level random effects term.

Inclusion of the autoregressive variable for abnormal breathing/dyspnoea 2 weeks previously was significantly associated with increased disease risk (adjusted OR 2.3, P = 0.041) in the transferrin D model and was marginally associated with an increased risk of similar magnitude (adjusted OR 2.1, P = 0.068) in the transferrin F2 model. As discussed for the modelling of nasal discharge, in order to best control for temporal association between outcomes without unnecessary reduction of the sample size, the autoregressive variable for the week previously was included *a priori* in the final model, even though it was itself not significantly associated with the outcome.

Examination graphically of how the risk of abnormal breathing/dyspnoea changed with increasing $log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes (Figure A2.12c) showed there was a satisfactory, although not perfect linear relationship between the logit of disease risk and $log_{10}$ cfu/ml *S. zooepidemicus.* Abnormal breathing/dyspnoea was, therefore, modelled, using the continuous variable of $log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes. Results showed that an incremental increase in $log_{10}$ cfu/ml *S. zooepidemicus* was associated with a significant increase in risk of abnormal breathing/dyspnoea in the transferrin D model (adjusted OR 1.3 per $log_{10}$ cfu/ml increase, P = 0.017) and a marginally significant increase in risk in the transferrin F2 model (adjusted OR 1.2 per $log_{10}$ cfu/ml increase, P = 0.076).

Binary variables for possession of either of 2 transferrin haplotypes (type D in Model 1 and type F2 in Model 2) were strongly significantly associated with abnormal breathing/dyspnoea. The direction of the effect of each of these variables was the same as had been observed in previous linear regression analyses of aggregated clinical outcome

scores and for logistic regression analysis of coughing. Possession of the transferrin D haplotype significantly reduced the risk of abnormal breathing/dyspnoea (adjusted OR=0.2, P=0.005) whereas possession of the F2 haplotype significantly increased the risk (adjusted OR=5.7, P=0.003).

Examination of pony-level variance estimates and their standard errors showed that they were likely to be at least marginally statistically significant in the 2 abnormal breathing/dyspnoea models, depending on the estimation method used. The MLE and second order PQL RIGLS estimates (β: S.E.β ratio ≥ 1.99) were higher than those of the second order PQL IGLS estimates (β: S.E.β ratio ≤ 1.71). As with other analyses the variable was retained *a priori* to account for the hierarchical structure of repeated measures data within ponies in the dataset. No statistically significant interaction terms were identified.

Figures A2.17 and A2.18 represent caterpillar plots of the pony level residuals for the final abnormal breathing/dyspnoea models calculated by RIGLS methods, which include transferrin D and F2 haplotypes, respectively.

Examination of the RIGLS pony level residuals and their 95% confidence intervals for the final abnormal breathing/dyspnoea model including transferrin D haplotype showed that the confidence intervals for 2 ponies either did not include zero (pony 29) or just included zero (pony 28). The residual for pony 29 had a positive value indicating that this animal had more abnormal breathing/dyspnoea than was predicted by the model. Examination of data (Figure A2.6) revealed that pony 29, which as well as being positive for transferrin D haplotype (by virtue of being DF2 phenotype) also lacked positive autocorrelated outcomes from 2 weeks previously and had relatively low $\log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes on 3 occasions when there was abnormal breathing/dyspnoea. Pony 28, with the largest negative value residual, did not possess

transferrin D haplotype and had no abnormal breathing/dyspnoea, but had high $log_{10}$ cfu/ml *S. zooepidemicus* for several weeks of the study.

Examination of the RIGLS pony level residuals and their 95% confidence intervals for the final coughing model that included transferrin F2 haplotype showed that the confidence intervals for only one pony (pony 23) did not include zero. The residual for pony 23 also had a positive value indicating that this animal had more abnormal breathing/dyspnoea than was predicted by the model. Examination of data (Figure A2.6) revealed that pony 23 was negative for the F2 haplotype (by virtue of being OH2 phenotype), lacked positive autocorrelated outcomes from 2 weeks previously on 3 occasions and had relatively low $log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes.

SMLN enlargement

The final logistic regression model for SMLN enlargement included the autoregressive variable for clinical outcome the previous week, vaccine group category and a pony-level random effect term.

The autoregressive variable for SMLN enlargement the previous week was significantly associated with increased risk of enlarged lymph nodes in the current week (adjusted OR 4.5, $P<0.001$). This variable was important as it completely confounded the significant associations with *S. zooepidemicus* infection in the trachea.

The other significant variable in the final logistic regression model of SMLN enlargement was that of vaccine group. Results showed that ponies that were introduced later were at significantly greater risk of SMLN enlargement compared to vaccinated animals (adjusted OR 7.4, $P = 0.014$) but that ponies that received placebo were at no significantly increased risk. No statistically significant interaction terms were identified.

There was evidence from the relatively larger coefficient estimates compared to their standard errors (i.e. β: S.E.β ratio ≥ 2.1) that for the SMLN model the random effect variance was likely to be significantly >0.

Examination of the RIGLS pony level residuals and their 95% confidence intervals for the final SMLN enlargement model (Figure A2.19) showed that the confidence intervals for only one pony (pony 23) did not include zero. The residual for pony 23 had a positive value indicating that this animal had more SMLN enlargement than was predicted by the model and the main reason for this was that it was in the placebo and not the late introduction vaccine group category.

# CHAPTER 9

# DISCUSSION

## 9.1 Discussion of overall study aims and design

This was a randomised, blinded, placebo-controlled study to evaluate an experimental inactivated, multivalent, adjuvanted bacterial vaccine in young Welsh Mountain ponies suffering natural respiratory disease.

The study had been conceived after several years of repeated clinical observations in young Welsh Mountain ponies brought to the AHT shortly after weaning for use in various studies, particularly those involving trials of respiratory virus vaccines. It was consistently noted by AHT clinicians and scientists that these young ponies suffered a considerable burden of clinical respiratory disease, characterised by mucoid nasal discharge, coughing and in some instances pyrexia, dyspnoea and pneumonia. This disease syndrome would frequently persist for many months in groups of animals that were kept predominantly outdoors at pasture.

Animals that were particularly badly affected were often treated for pneumonia with anti-inflammatory and antimicrobial therapy. The large proportion of positive responses to treatment seen in these cases, along with *post mortem* investigations in those ponies that died despite treatment, was consistent with this disease syndrome being closely associated with bacterial infection of the airways, which were affected with mucoid inflammatory exudate. The bacterial species most commonly isolated from the lungs of affected ponies included *S. zooepidemicus, S. pneumoniae, A. equuli, Pasteurella* spp. and *B. bronchiseptica.* Together with the detailed studies conducted by researchers at the AHT into inflammatory airway disease in young racehorses in training (Burrell, 1985; Burrell *et al.*, 1986b; Wood *et al.*, 1993a; Burrell *et al.*, 1994; 1996; Wood *et al.*, 1997b; Wood, 1999), these observations suggested that non-Thoroughbreds maintained as a herd outdoors

underwent a clinically similar form of respiratory disease as housed, young Thoroughbred racehorses in the early part of their training careers. On the basis of epidemiological studies in Thoroughbreds and the observation in ponies, a multivalent vaccine containing 2 strains each of *A. equuli* and *S. zooepidemicus* was developed by a commercial collaborator for evaluation in an appropriately conducted clinical trial. Observations in several challenge studies (Blunden *et al.*, 1994) (N. Chanter, unpublished observations) suggested that experimental infection with the more common presumptive pathogens such as *S. zooepidemicus* and *A. equuli,* was most often confounded by natural disease. A natural disease challenge was considered the only available and appropriate infectious setting for the vaccine trial, particularly due to the predictable occurrence and nature of the disease process in ponies.

In considering the most appropriate method of evaluating the vaccine within a natural disease challenge setting, it was deemed important that ponies should be vaccinated *prior* to the time of highest risk of natural disease challenge. This had been frequently observed to be soon after the stress of weaning, mixing with other ponies and hence their infections, and transportation to the AHT from different locations. Ponies in this study that were assigned to receive either vaccine (n=12) or placebo (n=12) were, therefore, vaccinated immediately prior to weaning and before mixing and transportation with other ponies (n=5) from geographically distinct areas. In order to try and minimise the amount of natural infection transmitted between ponies prior to vaccination, ponies receiving vaccine or placebo were chosen from as small a geographical area as possible, in this case the same Welsh hillside.

It was considered essential that a blinded, randomised and controlled study design was used in the evaluation of effectiveness of vaccination in reducing respiratory disease, in order to avoid observer bias. Treatment allocation was done randomly using a random permuted block design. In order to maximise the blinding, a placebo control was used,

which like the vaccine, was injected in the middle third of the neck on the left-hand side in each pony. Therefore, administration of a placebo in this study rather than non-administration had the advantage that it would not subsequently be obvious from any signs of injection such as blood leakage or swelling in this area which animals had received vaccine and which had not (i.e. signs caused by the action of intramuscular injection, irrespective of likely reaction to bacterial vaccine components). In addition, the use of a placebo consisting of alum adjuvant *without* the bacterial antigens would ensure that any significant differences observed were, therefore, more likely attributable to the antigenic portion rather than from any effect from adjuvant.

In considering the endpoints of interest in this study it was recognised that repeated measures in individual animals would be warranted because of the dynamic and time-varying nature of respiratory disease in young horses. In addition, detailed clinical, endoscopic and microbiological investigations were required at each sampling point to best characterise the disease and infection status of the animal over an extended period of time.

For practical logistical reasons, weekly intervals were selected for repeated sampling of the airways and summary clinical measures were produced to correspond with these. Each pony was examined clinically in detail on 2 occasions during each week and this was combined with endoscopic assessment of mucus in the trachea, collection of tracheal wash and nasopharyngeal swabs on a single occasion each week. Results of microbiological culture of wash and swab samples were then conducted immediately and completed before the next sampling occasion.

To our knowledge this was the first intensive and repeated clinical and microbiological investigation of naturally occurring respiratory disease in such young non-Thoroughbred horses combined with a clinical trial of a bacterial vaccine.

Although a detailed longitudinal study involving repeated sampling using endoscopy, tracheal wash and nasopharyngeal swabbing had been conducted in Thoroughbreds in

training (Wood, 1999), the current study used more intensive weekly sampling over a shorter period of 10 weeks compared to monthly sampling over a longer period. The current study therefore allowed a detailed 'snapshot' of the dynamics of respiratory disease in recently weaned Welsh Mountain ponies maintained outdoors, shortly after weaning, mixing and transporting. It was very important that the likely strong correlation between repeated samples from the same individual animals was considered carefully in the analysis of data from this study and this is discussed in detail later.

## 9.2 Evaluation of vaccine efficacy

The main purpose of this study was to evaluate the efficacy of a bacterial vaccine under conditions of natural respiratory disease in young Welsh Mountain pony foals. The vaccine was an inactivated, alum adjuvanted vaccine containing each of 2 strains of *S. zooepidemicus* and *A. equuli*, both recognised previously as being strongly associated with lower respiratory disease in young horses (Wood *et al.*, 1993a; Ward *et al.*, 1998; Chapman *et al.*, 2000; Christley *et al.*, 2001b).

Preliminary univariable analyses of pony-level summary data indicated that there was no evidence for statistically significant differences in clinical respiratory disease or infectious burdens between pony groups that received vaccine, placebo or were introduced later. This was in contrast to an apparently strong effect of transferrin haplotype. It had been Dr. Chanter's impression immediately after the study that ponies tended to fall into 2 clinically distinct groups, those with severe respiratory signs and high infectious burdens and those that were much less severely clinically affected and had lower infectious burdens. Formal analysis of pony-level data apparently confirmed this clinical impression and suggested that it was not attributable to vaccine but appeared closely linked to the genetically determined transferrin haplotype of the ponies.

Closer examination of observation-level data, taking particular care to control for temporally correlated outcomes within individual ponies and random variation between them, indicated that there was evidence for statistically significant differences in some individual clinical signs between the vaccine groups after controlling for other significant factors. Consistent with the pony-level analyses, however, there was no evidence for a significant effect from vaccination when aggregated clinical and airway inflammation scores were used as outcomes.

Among individual clinical outcomes, coughing and SMLN enlargement demonstrated statistically significant differences between vaccine groups when examined in univariable and multivariable analyses. Compared to ponies that were adminstered vaccine the placebo group had significantly more coughing and the late introduction group significantly more SMLN enlargement.

After controlling for the effects of coughing the previous week, *S. zooepidemicus* infection in the trachea, transferrin haplotype and random variation between ponies, ponies that received placebo remained at significantly increased risk of coughing compared to those that received vaccine. Although the estimate of risk of coughing varied between final logistic regression models that included different transferrin haplotypes (transferrin D model OR 4.5; P = 0.007 & transferrin F2 model OR 7.1; P = 0.002), confidence limits overlapped and results indicated that vaccinated ponies were significantly less likely to cough than those that received placebo.

It has been observed previously that coughing is an insensitive but specific measure of respiratory disease in horses (Burrell *et al.*, 1996; Head & Wood, 2001) The finding that cough was the least prevalent clinical sign (cough score summary, Table A2.1) in this study was consistent with coughing being a relatively poor predictor of respiratory disease. It is unclear at the moment what mechanism of action might be responsible for the significant reduction in coughing among vaccinated ponies compared to adjuvant (placebo)

administered controls, but this finding suggests there may be some link between bacterial infection of the airways and coughing.

After controlling for SMLN enlargement the previous week and random variation between ponies there was an increased risk of SMLN enlargement among the late introduction group (OR 7.4; P = 0.014) and no significant difference in risk in the placebo group (OR 1.4; P = 0.608) compared with vaccinated ponies. Vaccination did not significantly affect SMLN enlargement but there was a difference between the late introduction group and the other ponies with respect to SMLN enlargement. It is not clear to what specific factors this difference may have been attributable, although it is likely that differences in disease and management between these groups prior to the study.may have been important.

## 9.3 Discussion of findings on naturally occurring respiratory disease

As well as providing a means of evaluating the efficacy of a killed bacterial vaccine this study also provided an opportunity to make detailed clinical and microbiological observations on naturally occurring respiratory disease in recently weaned Welsh Mountain ponies. Multivariable analyses of observation-level data have provided an insight into various risk factors associated with this respiratory disease.

### 9.3.1 Risk factors associated with individual clinical signs

In this study the same observer (Dr. Neil Chanter), who remained blinded as to the vaccination status of ponies until after completion of the study, conducted all clinical examinations. Clinical examinations were reasonably detailed and assessed 5 separate clinical parameters (nasal and ocular discharges, coughing, abnormal breathing/dyspnoea and SMLN enlargement), with allocation of a score according to the severity of each sign. In order that results of respiratory sampling that was conducted only once a week, could be

matched with results of twice weekly clinical examinations, mean weekly scores for individual clinical parameters were calculated. The weekly average rather than, for example, the maximum score from the week, was considered the most suitable and discriminatory way of representing the overall weekly burden of clinical respiratory signs. Using this method, ponies that had maximum clinical severity at both examinations would score a higher weekly average score than a pony that had only shown the maximum severity at one examination.

In this study the clinical sign of ocular discharge was not well predicted by any of the factors examined, including autoregressive variables. This was consistent with this sign not being an important part of the respiratory disease syndrome demonstrated by young Welsh Mountain ponies. Given that ponies were fed from elevated hay racks it is probable and consistent with previous observations in other ponies managed in the same way that the ocular discharges observed were due to irritation from airborne dust released during communal feeding rather than respiratory disease *per se*.

*9.3.1.1 Infections*

Among nasopharyngeal infections, isolation of *B. bronchiseptica,* was found to be associated with a significantly increased risk of nasal discharge after controlling for other factors. *Bordetella bronchiseptica* has been reported from cases of respiratory disease in young horses previously, including from nasal discharges (Cockram *et al.*, 1981; Bayly *et al.*, 1982; Vandevenne *et al.*, 1995). Although previous case reports have suggested a primary role for *B. bronchiseptica* in respiratory disease in young horses, particularly shortly after weaning and there was a notable temporal clustering of isolations from ponies during this study (weeks 3-5), there was actually very little evidence to show that this infection was associated with signs of respiratory disease.

After controlling for the effects of other factors, infection of the trachea with *S. zooepidemicus* was found to be significantly associated with an increased risk of nasal discharge, coughing and abnormal breathing/dyspnoea. There was evidence with all 3 signs of a dose response relationship between the risk of the sign and the level of *S. zooepidemicus* infection in the trachea as measured by $\log_{10}$ colony forming units per ml of tracheal wash. Tracheal infection with *S. zooepidemicus* has been previously shown to demonstrate a dose dependent association with increasing risk of coughing (Christley *et al.*, 1999b; 2001b) and inflammatory airway disease (IAD) (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood, 1999; Chapman *et al.*, 2000) as defined by increased tracheal mucus and airway neutrophilic inflammation.

The finding of significant association between tracheal *S. zooepidemicus* infection and increased risk of nasal discharge appears contrary to the results of Burrell *et al.* (1996), who found no such association in racehorses in training, although they did show a significant association with infection with *S. pneumoniae*. Differences in the age of horses, definitions of disease, frequency of sampling and study duration may account for these apparently dichotomous findings, and the non-significant findings of Burrell *et al.* (1996) may have been due to a small sample size. In a follow-up longitudinal study of respiratory disease in Thoroughbreds, Wood (1999) recognised that concurrent IAD was a significant risk factor for nasal discharge and that adjusting for IAD accounted for the effects of infections such as *S. zooepidemicus,* that were associated with both IAD and nasal discharge. In the current study extremely detailed clinical and endoscopic examinations were performed concurrently and analyses for individual clinical signs were conducted using tracheal and nasopharyngeal infections as risk factors. Although nasopharyngeal isolation of *S. zooepidemicus* was associated with increased risk of nasal discharge in univariable analysis, it was not statistically significant in multivariable analyses. Results here indicate that tracheal *S. zooepidemicus* infection of the lower airway was a significant risk factor for

nasal discharge and should be considered consistent with the observations of Wood (1999). This was probably because this infection was associated with increased tracheal mucus and inflammation, as in young Thoroughbreds, which after natural tracheal mucociliary clearance appeared as a nasal discharge. This would suggest that at least in the young Welsh Mountain ponies studied here that the distinction between IAD and nasal discharge is not as clear-cut as that defined in Thoroughbreds in training by Wood (1999). This might be consistent with an age-related change in the relationship between *S. zooepidemicus* associated tracheal and nasal pathology and immunology (i.e. mucoid inflammatory exudate in response to infection). This may reflect differences in the immunological processes at these different levels in the equine respiratory tract at different stages. This in turn may help explain age-related clinical phenomena such as pharyngeal lymphoid hyperplasia, the severity of which has been shown to have an inverse correlation with age (Burrell, 1985) and the marked reduction in prevalence of nasal discharge between yearling Thoroughbreds and older horses (Wood *et al.*, 1998; Wood, 1999).

The finding of a dose response relationship between *S. zooepidemicus* in tracheal washes and the clinical sign of abnormal breathing/dyspnoea is consistent with this bacterium being the most frequently isolated organism from cases of pneumonia and pleuropnemonia, which frequently demonstrate abnormal patterns of breathing (Chaffin *et al.*, 1994a; 1994b; Raidal, 1995).

No individual infections were found to be significantly associated with SMLN enlargement during the 10 weeks of intensive examinations in this study, although as described previously, there was a significant difference in this outcome between the late introduction ponies and all others, possibly due to differences in earlier infections.

### *9.3.1.2 Clinical signs in previous weeks*

For all individual clinical signs with the exception of ocular discharge, presence of the sign in an earlier week significantly increased the risk of an animal having the same sign in the current week, although nasal discharge was not consistent with other signs because only discharge 3 weeks previously was significant. The variables for signs in previous weeks were referred to in analyses as autoregressive variables and in this study corresponded to signs one, 2 and 3 weeks previously to that being considered (current).

In presenting results of multivariable analyses for individual clinical signs, it was considered appropriate to include at least the autoregressive variable for the sign one week previously. Where appropriate and to avoid unnecessary loss of effective sample size, autoregressive variables for earlier weeks were only included when they at least approached statistical significance.

It is of note that for nasal discharge and abnormal breathing/dyspnoea that only the autoregressive variables corresponding to 3 and 2 weeks previously, respectively, were significantly associated with the presence of the clinical sign in the current week. This most probably reflected the somewhat intermittent nature of these clinical signs between successive weeks (as seen in the clinical sign profiles outlined in Figure A2.6). It is not clear whether this truly reflected repeated occurrence of clinical signs of short duration or a variation in severity of signs associated with the same prolonged clinical episode. The inclusion of other variables and particularly pony-level random effects terms in the final logistic regression models for individual clinical signs significantly altered the level of significance and size of effects of the autoregressive variables compared to univariable analyses. This reflected confounding between temporally related outcomes accounted for by inclusion of autoregressive fixed effects parameters and pony-level random effect terms, which accounted for random variation between individual ponies.

Previous studies of equine respiratory disease using repeated measures are extremely limited (Morley, 1995; Wood, 1999). Where a longitudinal study design has been conducted previously in Thoroughbreds in training (Wood, 1999), the sampling interval (one month) and duration of the study (3 years) were both extended compared to the current study. In the longitudinal study by Wood (1999) autoregressive variables for IAD and mucus in the trachea the previous month were included in final regression models and demonstrated at least marginal significance, but a final model for nasal discharge did not include a significant autoregressive variable. It was, therefore, not surprising that in this study where clinical examination and sampling was conducted at shorter weekly intervals, that autoregressive variables were significant predictors of clinical signs.

*9.3.1.3 Transferrin haplotype*

Consistent with the results of the preliminary univariable analyses of pony-level summary data, coughing and abnormal breathing/dyspnoea were both significantly associated with specific transferrin haplotypes possessed by ponies. As with the preliminary analyses 2 final models were produced for these 2 outcomes, corresponding to the separate inclusion of transferrin D and F2 haplotypes.

There was consistency of direction of the effect of each transferrin haplotype between the 2 different clinical signs. Possession of transferrin D haplotype was associated with a significant protective effect whilst possession of the F2 haplotype was associated with a significantly increased risk of clinical signs. Results were also consistent with the pony-level analyses in that the size and significance of the protective effect associated with possession of the transferrin D haplotype were each slightly smaller than the corresponding values for the increased risk attributed to the F2 haplotype. This most likely reflected the influence of the 4 ponies that had DF2 phenotypes and as such acted to contribute to the effect of both haplotypes. As was seen in the pony-level data, ponies with transferrin DF2

phenotype generally suffered more severe clinical signs than other D haplotypes that were not also F2 haplotype. This resulted in the overall effect of the D haplotype being reduced compared with the F2 haplotype.

The implications of the findings of this study with respect to transferrin haplotype as a significant risk factor for equine respiratory disease and possible mechanisms of action are discussed in detail in the discussion of aggregated clinical scores.

*9.3.1.4 Pony-level random effects*

As well as the inclusion of autoregressive outcomes for earlier weeks, final models for individual clinical signs were presented with inclusion of a pony-level random effect term. This was in order to account for correlation between repeated observations from the same individual ponies, which were themselves assumed as having some random variability with respect to the outcomes of interest. Pony-level random effects were retained in all final models irrespective of whether they were considered to be themselves statistically significant as suggested by Mauritsen (1984) and adopted also by Wood (1999). This allowed the size and significance of other effects to be examined without the effects of clustering of repeated observations within ponies.

Examination of the coefficient estimates and their standard errors for the random effects terms in the final models for each of the clinical signs excluding ocular discharge, demonstrated variation in the likely significance of these terms and hence differences in the likely clustering of different signs within ponies. Consistent with transferrin haplotype being an important predictor of respiratory disease at the pony-level, its inclusion in models for coughing and abnormal breathing/dyspnoea apparently resulted in lower likely significance of random effects terms than in models for nasal discharge and SMLN enlargement, that did not include transferrin haplotype variables. This difference was particularly noticeable for models generated using second order PQL IGLS estimates in MLwiN software. This

suggested that the inclusion of particular transferrin haplotypes, which were fixed effects at the pony-level, was accounting for some of the random variation between ponies and was consistent with ponies of different haplotypes having different susceptibilities to coughing and abnormal breathing/dyspnoea.

### 9.3.2 Risk factors associated with aggregated clinical and IAD scores

#### *9.3.2.1 Use of aggregated scores*

Clinical scores

Two aggregated weekly clinical scores were used to quantify the overall severity of clinical respiratory disease for each pony during each week of the study. Both these scores were the sum of weighted individual clinical sign scores. Clinical score was derived from the sum of the average weekly scores for all 5 clinical signs with nasal and ocular discharges, coughing and abnormal breathing/dyspnoea each weighted equally with a maximum possible contribution of 2 points to the score. SMLN enlargement was considered less important than the other signs and as such only contributed a single point when present. The other aggregated score, which was derived from the weekly sums of coughing (C), abnormal breathing/dyspnoea (D), nasal discharge (N) and SMLN enlargement (S), was referred to as CDNS score and did not include a score for ocular discharge on the premise that this might not be a sign of respiratory disease *per se*. From analyses of data from individual clinical signs and comparisons of analyses using clinical and CDNS scores, this premise appears to have been well founded as ocular discharge was not strongly associated with plausible risk factors for respiratory signs and its exclusion from the aggregated scoring did not significantly alter results.

Aggregated clinical scores used in this study were derived from simple classifications of each clinical sign that with the probable exception of ocular discharge, were believed to be specific to and indicative of respiratory disease at particular levels of the respiratory

tract. Although in this trial there was a single observer, the clinical scoring that was adopted attempted to avoid subjective interpretation of parameters for which measures of severity could not be well defined such as demeanour, appetite and depression. These clinical parameters were, therefore, not used because the time spent observing animals was short, the presence of an observer might influence their behaviour and assessment of appetite in animals grouped together was very difficult.

In this study, multilevel models with inclusion of autocorrelated outcomes were used to examine the relationships between disease outcomes and expoasures which clustered at 2 different levels within the data. However, alternative approaches could have been taken to examine the functional form of these relationships, including generalised additive models, orthogonal polymonials and time series with independent autocorrelation, which are available in various different software packages such as SAS, S-Plus and MLwiN (Grohn *et al.*, 1999; Green *et al.*, 2002; Hirst *et al.*, 2002; Pinchbeck *et al.*, 2002).

Airway inflammation

In this study a 9-point scoring system was used based on an expanded version of the 3-point system used in earlier studies conducted by the AHT (Whitwell & Greet, 1984; Wood *et al.*, 1993a; Burrell *et al.*, 1996) and similar to that described by Chapman *et al.* (2000). It was considered that this expanded scoring system, because it used 0 - 3 scores for each of the contributory elements (tracheal mucus, tracheal wash smear cell density and neutrophil proportion) rather than single scores as described by Whitwell & Greet (1984), was likely to be more discriminating as to the severity of airway inflammation. In addition, the use of nucleated cell counts, which have been found to be problematical, particularly when done automatically because of the clumping of cells in mucoid exudate, was replaced by semi-quantitative assessment of the density of cells on cytological smears. This was

considered likely to be a more reproducible measure of the absolute number of cells present in tracheal washes.

In considering airway inflammation as a disease outcome it is important to also consider possible causal relationships ain conjunction with possible causal pathway effects between risk factors and these outcomes. By way of example, tracheal mucus forms part of the scoring system for airway inflammation in this study and has itself been used as an outcome in previous studies (Wood, 1999). Although there is multiple evidence for a causal association between mucus in the trachea and bacterial infections with a restricted number of specific bacterial species (Wood & Chante, 1994), it has been proposed that tracheal bacterial infection is on the causal pathway of tracheal mucus because mucus acts to trap bacteria and numbers are increased with reduced mucociliary clearance (N. E. Robinson – personal communication).

*9.3.2.2 Infections*

No specific nasopharyngeal bacteria were significantly associated with any aggregated clinical and airway inflammation scores, although 3 bacterial species isolated from tracheal washes were.

For clinical, CDNS and airway inflammation score models, *S. zooepidemicus* and non-haemolytic *Streptococcus* spp. were identified as significant predictors when included as linear and quadratic terms, respectively. This indicated that higher scores for all 3 outcomes were associated with higher colony numbers of *S. zooepidemicus* in tracheal washes and was consistent with previous studies of both clinical disease (Burrell *et al.*, 1994; Christley *et al.*, 1999b; 2001b) and airway inflammation (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood, 1999; Chapman *et al.*, 2000). The relationship between outcome scores and non-haemolytic *Streptococcus* spp. in this study appears less straightforward than for *S. zooepidemicus* as it is best fitted by a quadratic function (Figure 8.4). This function

indicated that the majority of counts of non-haemolytic *Streptococcus* spp. ($10^2$ - $10^6$ cfu/ml) were associated with no difference or a decrease in outcome score and only the extremes of no bacteria isolated and very high colony counts were associated with slightly higher scores. This sort of distribution was consistent with disease being associated with mixed infections with large numbers of other bacteria in the absence of non-haemolytic *Streptococcus* spp. in tracheal washes. This finding appeared consistent with observations in some species of bacteria (*Staphylococcus* and *Acinetobacter* spp.) in the case control study of clinical respiratory disease in Thoroughbreds in that their presence, or at least their identification, was more likely in the absence of disease and isolation of other bacteria in large numbers.

In models for clinical and CDNS scores but not airway inflammation score, *Pasteurella* spp. in tracheal washes were also predictive of outcome when modelled as a quadratic function (Figure 8.3). This function indicated that in this study the majority of counts of *Pasteurella* spp. ($<10^6$ cfu/ml) were associated with no difference in outcome scores and only the higher colony counts, which were relatively small in number, were associated with higher scores. This finding appeared somewhat inconsistent with previous observations in Thoroughbreds in training in which *Pasteurella* spp. have been shown to be strongly associated with clinical signs (Burrell *et al.*, 1994; Christley *et al.*, 1999b; Christley *et al.*, 2001b) and airway inflammation (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood, 1999; Chapman *et al.*, 2000). However, this was a very restricted study with respect to numbers of ponies and period of study and important bacterial species may have been missing.

With all infections taken together, results were suggestive that in this group of young Welsh mountain pony foals the predominant infection associated with clinical and airway inflammation was *S. zooepidemicus* isolated from the trachea.

### *9.3.2.3 Scores in previous weeks and pony-level random effects*

As with most individual clinical signs, aggregated outcome scores from at least the previous week were significantly predictive of outcome score in the current week and inclusion of a pony-level random effect term allowed sample sizes to be maximised as autoregressive variables from earlier weeks were no longer significant and were dropped from analyses.

In the multilevel modeling, pony-level random effects were retained in all final models, whether they were statistically significant or not. In the final clinical score model the pony-level random effect term appeared statistically significant and there was an intra-pony correlation of 14.4% for both models that contained transferrin D or F2 haplotypes as a fixed effect parameter. With exclusion of ocular discharge and use of CDNS score as the outcome, the significance of the pony-level random effect term and the intra-pony correlation (12.7%) were both reduced compared to the clinical score model. It was possible that this might have been due to the reduction in parameters contributing to CDNS score, making all scores more similar, although as expected this led to both a reduction in observation-level as well as pony-level variances with the reduction in observation-level variance being greater.

The final multilevel model for airway inflammation was equivalent to the multiple linear regression model as it did not have statistically significant pony-level random effects and the intra-pony correlation was consequently very small. It is likely that the use of a 9-point inflammation score from a single sampling occasion per week was not satisfactorily discriminating to demonstrate any residual pony-level random variation when all significantly predictive variables were included. This would have arisen if the range of airway inflammation scores in individual animals was limited over the duration of the study as a consequence of a high prevalence of airway inflammation. A high prevalence would be expected if the duration of airway inflammation was long relative to the 10 consecutive

weeks of sampling. Indeed in these recently weaned ponies, >80% (252/314) of samplings demonstrated inflammation scores of ≥5/9. In a previous study of inflammatory airway disease in Thoroughbreds, the mean duration of disease defined on the basis of similar criteria was almost 8 weeks, not far short of the consecutive sampling period in this study (Wood *et al.*, 1998; Wood, 1999).

*9.3.2.4 Transferrin haplotype*

Results of both pony-level and observation-level analyses demonstrated clear evidence that, after controlling for other significant factors, different transferrin haplotypes were associated with significant differences in aggregated clinical scores, particularly when CDNS score was used. As discussed previously, both the pony- and observation-level analyses were consistent in demonstrating the direction of effect for the 2 significant haplotypes (possession of transferrin D haplotype was protective and the F2 haplotype was associated with increased risk). If this was manifested by differences in the ability of different transferrin haplotypes to relinquish iron to bacteria by either TBPs or other mechanisms, then it would be reasonable to predict that iron was more readily sequestered from F2 haplotype transferrin than D. The finding that DF2 phenotypes behaved more like F2 haplotypes than D was consistent with this hypothesis. However, the small numbers (n≤5) of animals of specific phenotypes precluded the meaningful investigation of differences between haplotypes other than D or F2 or specific haplotype combinations (phenotypes).

Results, particularly those from the pony-level analyses, suggested that in this study the significant differences in clinical outcomes observed between ponies with different transferrin haplotypes were associated with differences in infectious outcomes with *S. zooepidemicus* rather than either *Pasteurella* or *Actinobacillus* spp.. The specific mechanism by which transferrin haplotype is related to differences in *S. zooepidemicus*

infection in ponies is not known at this stage. It is possible that there is a direct link between pathogenesis of *S. zooepidemicus* infection and transferrin haplotype similar to that identified for *S. intermedius* by Brochu *et al.* (1998). Alternatively, the association between transferrin haplotype and *S. zooepidemicus* may be evidence of a different mechanism that is manifested through a gene closely linked to the equine transferrin locus. Further studies specifically aimed at characterising iron binding in Streptococci of clinical importance in horses (*S. equi*, *S. zooepidemicus* and equine isolates of *S. pneumoniae* capsule type 3) are warranted. This research, in conjunction with the imminent completion of the sequencing of the *S. equi* genome, may provide significant benefits in development of effective equine bacterial vaccines.

Whatever the mechanism of action the results of this study appear to be novel and as such require confirmation with respect to their repeatability in other groups of horses. To this end routine prospective data collection has been initiated at the AHT in order to systematically monitor clinical respiratory disease in Welsh Mountain pony foals within a few months of their arrival from Wales. This data will be analysed for evidence of significant differences in clinical respiratory disease between ponies of different transferrin haplotypes and with sufficient numbers of animals it is hoped to better elucidate differences between ponies with specific transferrin phenotypes.

In analyses of clinical and CDNS scores, the use of multilevel modelling incorporating random variability between repeatedly sampled ponies and including addition of pony-level fixed effect variables such as sex, vaccine group and transferrin haplotype, permitted examination of these variables in explaining variation between ponies. Results of model building demonstrated that inclusion of transferrin haplotype but not sex or vaccine group, which each varied between ponies but not between observations in the same pony, did significantly account for some but not all of the variation between individual ponies. As a proportion of the between pony variation was not explained by transferrin haplotype, this

demonstrates that there must be other horse-level factors such as different major histocompatibility complex (MHC) classes that may also account for variation in disease outcomes between individual animals.

Recent analogous data from Thoroughbreds

As far as the author is aware these are the first data that suggest significant differences in clinical respiratory disease associated with bacterial infections between equids on the basis of equine transferrin haplotypes, a genetically determined trait. If this is a true effect then the results seen here should be supported by analogous data from other equine populations.

To this end recent results of analyses examining the effects of transferrin haplotypes on IAD in young racehorses have also demonstrated a significantly protective effect with possession of the D haplotype (R. Newton, unpublished observations). Despite marked differences in the 2 study populations with respect to breed and age of animals, definitions of respiratory disease and interval and duration of sampling, this was consistent with the direction of the effect of this transferrin haplotype for clinical respiratory disease in Welsh Mountain pony foals.

This pony study identified significant and opposing effects from transferrin D and F2 haplotypes on infectious measures and there was evidence from the Thoroughbred data for similar directions of association with these 2 haplotypes. For transferrin haplotype D, infectious variables were reduced overall in horses possessing this haplotype which was consistent with the direction of effect seen in ponies. Observations from racehorses possessing haplotype F2 had significantly increased counts of *S. pneumoniae*, *S. equisimilis* and *M. equirhinis*, which was consistent with the direction of effect seen in ponies and opposite to that observed for haplotype D.

Multivariable logistic regression analyses of IAD in Thoroughbreds demonstrated at least marginal significance for transferrin haplotype D with inclusion of other significant variables including different infections.

Additional horse-level analyses were conducted to investigate whether there were significant differences in the prevalence and duration of IAD between horses with and without transferrin haplotype D. Results showed a significant difference in duration of IAD episodes (significantly reduced duration with haplotype D), which would account for some of the significant difference in overall IAD prevalence, although there was still a marginally significant difference between prevalence of separate IAD episodes. This suggested that horses with transferrin haplotype D suffered less IAD episodes and those that they did suffer were on average of shorter duration than horses that did not possess this haplotype.

# SECTION 4

# THE MOLECULAR EPIDEMIOLOGY OF *STREPTOCOCCUS ZOOEPIDEMICUS* INFECTION IN WELSH MOUNTAIN PONY FOALS

# CHAPTER 10

# INTRODUCTION

## 10.1 Background

As already discussed epidemiological studies have consistently demonstrated significant associations between tracheal *S. zooepidemicus* infection and IAD, with or without coughing, in racehorses (Wood *et al.*, 1993a; Burrell *et al.*, 1996; Wood, 1999; Chapman *et al.*, 2000; Christley *et al.*, 2001b). In addition, previous observations in Thoroughbred (Hoffman *et al.*, 1993c) and Welsh Mountain pony foals (AHT unpublished observations) have identified *S. zooepidemicus* associated with both clinical and endoscopically visible respiratory disease. From this evidence it is clear that young horses suffer prolonged and repeated episodes of respiratory disease associated with lower airway infection with *S. zooepidemicus* and higher counts of *S. zooepidemicus* in tracheal washes are associated with more severe respiratory disease (Wood *et al.*, 1993a; Wood, 1999).

However, as *S. zooepidemicus* is not a homogenous bacterial species of a single subtype but comprises many different subtypes (Moore & Bryans, 1969; Jorm *et al.*, 1994; Chanter *et al.*, 1997; Walker & Timoney, 1998), it is not clear how the prolonged and repeated nature of respiratory disease in young horses relates to variations in infections with different subtypes. In order to address this issue, stored frozen isolates of *S. zooepidemicus* from tracheal washes and nasopharyngeal swabs from the study of natural respiratory disease in Welsh Mountain pony foals (Section 3) were re-cultured and typed by 2 separate PCR assays (Chanter *et al.*, 1997; Walker & Timoney, 1998). The typing was conducted as part of a project on the molecular epidemiology of *S. zooepidemicus* in horses funded by the Horserace Betting Levy Board (Project 673). A dedicated graduate technician with previous experience of PCR conducted the typing of pony isolates after optimising the assays. The results were initially collated and checked by the project's bacteriologist, Dr.

Neil Chanter, and then by myself immediately prior to formal statistical analyses, which are presented here.

## 10.2 Aims of the study

The first aim of the study was to type isolates of *S. zooepidemicus* from tracheal washes and nasopharyngeal swabs recovered from Welsh Mountain ponies suffering natural respiratory disease during an experimental bacterial vaccine trial through application of 2 different PCR typing techniques. As the PCR techniques are able to characterise up to 8 types of the 16S-23S RNA gene intergenic spacer (Chanter *et al.*, 1997) and up to 5 types of the M-protein hypervariable region (Walker & Timoney, 1998), together they can potentially identify up to 40 different types.

Using this typing information, additional aims of the study were:

i) to estimate the overall prevalence of different *S. zooepidemicus* types among the group of 29 Welsh Mountain ponies during the study,

ii) to assess the prevalence of the same types at different locations in the respiratory tract by comparing tracheal and nasopharyngeal samples,

iii) to assess the changes in prevalence of different types both overall and at the different sites in the respiratory tract (trachea and nasopharynx) over the period of the study,

iv) to investigate the association of specific *S. zooepidemicus* types with respiratory disease whilst controlling for the effects of other risk factors including the experimental bacterial vaccine, which contained antigens from 2 specific *S. zooepidemicus* types,

v) to assess whether there was evidence for clonal succession of types during natural respiratory infection by *S. zooepidemicus* in these ponies.

# CHAPTER 11

# MATERIALS AND METHODS

## 11.1 Laboratory methods

The bacteriology laboratory methods used to identify and quantify bacterial species in the pony study in both tracheal wash and nasopharyngeal swab samples have been described previously in Chapter 3 in Section 2.

### 11.1.1 Storage and re-isolation of *S. zooepidemicus* isolates

After 3 passages on streptococcal selective agar (BioMerieux) colonies of *S. zooepidemicus* were transferred from the agar to Eppendorf tubes containing 1.0 ml of sterile 25% (v/v) glycerol in Todd-Hewitt broth by immersion of a sterile cotton swab after streaking across the culture. Isolates were immediately stored frozen at –50°C until typing was conducted subsequently with subculture of the frozen stock on 5% (v/v) horse blood agar.

### 11.1.2 *S. zooepidemicus* DNA extraction for PCR

A single colony forming unit of the subcultured *S. zooepidemicus* isolate was picked from the agar plate using a sterile flamed loop and added to 25 µl Proteinase K buffer (1x Gene Amp Buffer [Perkin Elmer], 0.5% [v/v] Tween 20, 100µg/ml proteinase K [Sigma-Aldrich]) in a 1.5ml Eppendorf tube. Samples were incubated at 55°C for 30 minutes in a water bath, denatured by boiling for 5 minutes and centrifuged for 5 minutes at 10,000g to collect the supernatant fraction for immediate PCR.

### 11.1.3 Polymerase chain reaction (PCR) assays

Nine different PCR assays based on the 16S-23S RNA gene intergenic spacer (Chanter *et al.*, 1997) (4 reactions: A-D) and hypervariable region of the M-like protein (Walker & Timoney, 1998) (5 reactions: 1-5) of *S. zooepidemicus* were used on each isolate. The gene regions, primer sequences and product sizes for these PCRs are summarised in Table 11.1.

During the conduct of PCR assays on isolates from this study, it became clear that some isolates did not have PCR products from any of the 5 reactions for M-like protein hypervariable regions. In order to confirm that these isolates did indeed possess M-like proteins but that their hypervariable regions were distinct from those used in the existing reactions, a reaction using the forward SzPN and reverse SzPNC3 primers was additionally used (Walker & Timoney, 1998). Isolates that yielded product for this but not other reactions were classified as having untyped hypervariable regions (HV untyped, HVu).

The 16S-23S RNA gene intergenic spacer PCR was conducted by mixing 1µl of extracted sample with 2.5µl of 10x GeneAmp PCR buffer (Perkin Elmer), 0.5µl of 10mM dNTP mix, 1µl of each forward and reverse primer mix at 25pMol/µl and 0.1µl of Ampli*Taq* (Perkin Elmer) 5 units/µl and 18.9µl of sterile pure water.

The M-like protein hypervariable region PCR was conducted by mixing 1µl of extracted sample with 2.5µl of 10x GeneAmp PCR buffer II (Perkin Elmer), 3.5µl of 20mM magnesium chloride, 0.5µl of 10mM dNTP mix, 1µl of each forward and reverse primer mix at 25pMol/µl 0.1µl of AmpliTaq (Perkin Elmer) 5 units/µl and 15.4µl of sterile pure water.

For both reactions, the mixture was heated at 95°C for 10 minutes followed by 40 cycles of 94°C for 1 minute, 56°C for 2 minutes and 72°C for 2 minutes followed by a period of 7 minutes at 72°C.

**Table 11.1: Summary of gene regions, primer sequences and product sizes for typing of *S. zooepidemicus* isolates by 16S-23S RNA gene intergenic spacer and M-like protein hypervariable region PCRs**

| Primers | Region | Primer sequence | Type(s) | Product size (bp) |
|---|---|---|---|---|
| ***Intergenic spacer*** | | | | |
| **Reaction** | **A** | | **A1, C1** | **142, 311** |
| Forward primers | Reg1a | AAAAAGGAAGCACGTTTAGCG | | |
| mixture | Reg1a* | AAAAAGGAACACGTTTAGCG | | |
| Reverse primer | Reg5a/6a | CCGTCTGTTAGTATCCTGTTT | | |
| **Reaction** | **B** | | **B1, D1** | **261, 424** |
| Forward primers | Reg1a | AAAAAGGAAGCACGTTTAGCG | | |
| mixture | Reg1a* | AAAAAGGAACACGTTTAGCG | | |
| Reverse primer | Reg6b | ATCGACGATGTGTGCTTTAC | | |
| **Reaction** | **C** | | **A2, C2** | **145, 314** |
| Forward primer | Reg1b | AAAAAWGGAAGCATGTTTGGAAG | | |
| Reverse primer | Reg5a/6a | CCGTCTGTTAGTATCCTGTTT | | |
| **Reaction** | **D** | | **B2, D2** | **264, 427** |
| Forward primer | Reg1b | AAAAAWGGAAGCATGTTTGGAAG | | |
| Reverse primer | Reg6b | ATCGACGATGTGTGCTTTAC | | |
| ***Hypervariable region*** | | | | |
| **Reaction** | **1** | | **1** | **403** |
| Forward primer | SzPN | ACAAAAGGGGAATAAAATGGC | | |
| Reverse primer | HV1R | CTGATAGTTGTTTACCAGCA | | |
| **Reaction** | **2** | | **2** | **781** |
| Forward primers | HV2F | AGTCGTTCTTGAGCAGAAAA | | |
| mixture | HV2VAR | AGTTGTTCTTGAGCAAAAAA | | |
| Reverse primer | SzPNC3 | TTTACCACTGGGGTATA | | |
| **Reaction** | **3** | | **3** | **411** |
| Forward primer | SzPN | ACAAAAGGGGAATAAAATGGC | | |
| Reverse primer | HV3R | ATGTAAACCTCCACCTGATAA | | |
| **Reaction** | **4** | | **4** | **409** |
| Forward primer | SzPN | ACAAAAGGGGAATAAAATGGC | | |
| Reverse primer | HV4R | CCCACACGTTCAGGTGATAA | | |
| **Reaction** | **5** | | **5** | **780** |
| Forward primer | HV5F | ATCATTCGTMAACAAGGCCC | | |
| Reverse primer | SzPNC3 | TTTACCACTGGGGTATA | | |

### 11.1.4 Detection of PCR products

After PCR reaction cycling was completed, products were detected by electrophoresis of 3µl of sample mixed with 3µl sample buffer in 2% (w/v) agarose (Promega), 0.04 mol/l Tris acetate (pH 8.3), 0.001 mol/l ethylene diamine tetra acetic acid (Sigma Aldrich) in GNA 100 apparatus (Pharmacia) at 110 volts for 35 minutes. Product sizes were determined by comparison with the relative mobilities of the Gibco 1kb standards (Gibco). Gels were examined on an ultraviolet transilluminator after immersion in 0.5µg/ml ethidium bromide for 15 minutes.

## 11.2 Data

The structure of much of the data for this study has been described previously in detail in Chapter 7 in Section 3. In addition to these existing data, results of the PCR typing of *S. zooepidemicus* isolates from tracheal wash and nasopharyngeal swab samples were generated and collated to form a larger dataset.

### 11.2.1 *S. zooepidemicus* typing data

Individual binary variables were initially generated corresponding to each specific *S. zooepidemicus* type as defined by its intergenic spacer type, its hypervariable region type and whether it was from either a tracheal wash or nasopharyngeal swab sample. Observations corresponded to each week of the study for each pony (n=319). These *S. zooepidemicus* type binary variables were classified as either zero if *S. zooepidemicus* was not isolated from the sample or if isolated was of a different type to that of the variable, as one if the isolate was of the exact type corresponding to the variable and as missing if no sample or isolate for typing was available. Pooling of data as a baseline category when *S. zooepidemicus* was a different type or not isolated from the sample was done to maximise reference category size as very few samples did not have this bacterial species isolated.

Additional continuous variables were generated corresponding to the quantification of specific *S. zooepidemicus* types in tracheal washes. These were generated by multiplication of the binary type variable by the $\log_{10}$ cfu/ml of *S. zooepidemicus* in the tracheal wash, as described in Chapter 5. These variables again took the value of zero if *S. zooepidemicus* was not isolated from the sample or if isolated was of a different specific type to that of the variable and were missing if no sample or isolate for typing were available.

## 11.3 Statistical analyses

Data from the typing of *S. zooepidemicus* was initially examined descriptively in terms of the proportions of typed isolates of each specific type for each site of sampling (trachea and nasopharynx). In addition, the proportion of ponies possessing specific *S. zooepidemicus* types in each sampling site was also investigated in terms of temporal trends and differences between vaccine groups.

Data were also examined for any association between individual *S. zooepidemicus* types and measures of respiratory disease. These were based largely on the methods described previously, substituting the generic *S. zooepidemicus* variable for specific types. Due to limitations on the numbers of positive isolates of particular specific types, analyses were restricted to the 5 most prevalent *S. zooepidemicus* types.

### 11.3.1 Aggregated clinical sign and airway inflammation scores

#### *11.3.1.1 Univariable analyses*

Data were examined in the first instance to determine whether there were any statistically significant relationships between outcome scores (Clinical score, CDNS score and airway inflammation score) and each of the 5 most prevalent *S. zooepidemicus* types. Linear and polynomial regression as described in Chapter 7 in Section 3 were used to

examine separately the relationship of $\log_{10}$ cfu/ml of specific *S. zooepidemicus* types with the aggregated scores. The most suitable polynomial forms for fitting the data were evaluated using the FRACPOLY and COMPARE commands available for linear regression analysis in Stata5.0 software. The results of polynomial regression analyses represented the best fitting regression models containing 2 or more power terms tested from combinations of $x^{-2}$, $x^{-1}$, $x^{-0.5}$, $x^{0.5}$, $x^{1}$, $x^{2}$, $x^{3}$ and $\log_e x$ and including products of $\log_e x$ with all other powers including itself.

The Wilcoxon rank sum test was used to examine whether there were any statistically significant differences in aggregated clinical and airway inflammation scores between the presence and absence of specific *S. zooepidemicus* types on nasopharyngeal swabs. Where significant differences were identified, the direction of the difference in clinical outcome score was examined. All univariable analyses were conducted using Stata5.0 computer software (Stata Corporation).

#### *11.3.1.2 Multivariable linear regression modelling excluding pony-level random effects*

Multiple linear and polynomial regression modelling was conducted using Stata5.0 computer software in the same manner as that described in Chapter 7, but with inclusion of appropriate variables to best represent individual *S. zooepidemicus* types in place of the generic *S. zooepidemicus* term.

#### *11.3.1.3 Multilevel linear regression modelling including pony-level random effects*

MLwiN1.10 computer software was used to perform multilevel linear and polynomial regression modelling with inclusion of random effects. Analyses were conducted as previously described in Chapter 7 but with inclusion of appropriate variables representing individual *S. zooepidemicus* types instead of the *S. zooepidemicus* variable.

## 11.3.2 Individual clinical signs

### *11.3.2.1 Univariable analyses*

Data were initially examined for the univariable association of the probability of presence of each individual clinical sign (nasal and ocular discharge, coughing, abnormal breathing/dyspnoea and submandibular lymph node enlargement) with each of the 5 most prevalent *S. zooepidemicus* types by inclusion in simple ordinary logistic regression (OLR) models. Tracheal wash *S. zooepidemicus* types were examined as binary (type not isolated or isolated), ordered categorical (type not isolated, isolated at $<10^3$ $\log_{10}$ cfu/ml or isolated at $\geq 10^3$ $\log_{10}$ cfu/ml) and continuous ($\log_{10}$ cfu/ml) outcomes. The association between outcome and each explanatory variable was expressed as a beta estimate and standard error ($\beta$ & S.E. $\beta$), an estimated unadjusted (crude) odds ratio (OR) with 95% confidence intervals around the estimate and corresponding Wald $\chi^2$ P-value.

The risk of each outcome with $\log_{10}$ cfu/ml of *S. zooepidemicus* types in tracheal washes were examined graphically to assess the most suitable way to express the levels of these variables in further multivariable analyses. Where there was not a satisfactory linear relationship between the logit of outcome risk and $\log_{10}$ cfu/ml *S. zooepidemicus*, an appropriate categorical summary was examined for its suitability of fitting data.

### *11.3.2.2 Multivariable logistic regression analysis ignoring pony-level random effects*

Multiple logistic regression modelling was conducted as described in Chapter 7 using Egret computer software with inclusion of appropriate variables representing individual *S. zooepidemicus* types in place of the single, general term for *S. zooepidemicus*.

#### *11.3.2.3 Multivariable logistic regression analysis accounting for pony-level random effects*

Multilevel logistic regression modelling with inclusion of pony-level random effects, was performed as previously described using MLwiN1.10 and Egret computer software. Analyses were conducted with inclusion of appropriate variables representing individual *S. zooepidemicus* types.

# CHAPTER 12

# RESULTS

## 12.1 Description of *S. zooepidemicus* typing data

### 12.1.1 All *S. zooepidemicus* types

Table 12.1 summarises the numbers and proportions of samples, isolations and different types of *S. zooepidemicus* from tracheal wash and nasopharyngeal swab samples from repeated sampling of 29 Welsh Mountain pony foals.

**Table 12.1: Summary of numbers and proportions of samples, isolations and types of *S. zooepidemicus* from tracheal wash (TW) and nasopharyngeal (NP) swab samples**

| | TW samples | | NP swab samples | | All samples (TW+NP) | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| **Total samples** | **314*** | **100** | **319** | **100** | **633** | **100** |
| *S. zooepidemicus* not isolated | 20 | 6.4 | 40 | 12.5 | 60 | 9.5 |
| *S. zooepidemicus* isolated (% = prevalence) | 294 | 93.6 | 279 | 87.5 | 573 | 90.5 |
| *S. zooepidemicus* isolate not available for typing | 19 | | 16 | | 35 | |
| **Number of *S. zooepidemicus* isolates typed** | **275** | **100** | **263** | **100** | **538** | **100** |
| Isolates of 10 most prevalent types (Table 12.2) | 230 | 83.6 | 222 | 84.4 | 452 | 84.0 |
| Isolates of other types | 45 | 16.4 | 41 | 15.6 | 86 | 16.0 |
| Isolates of types from both TW and NP | 262 | 95.3 | 254 | 96.6 | 516 | 95.9 |
| Isolates of types from TW or NP only | 13 | 4.7 | 9 | 3.4 | 22 | 4.1 |
| **No. of *S. zooepidemicus* types represented** | **33** | **100** | **27** | **100** | **39** | **100** |
| Types isolated from both TW and NP | 21 | 63.6 | 21 | 77.8 | 21 | 53.8 |
| Types isolated from TW or NP only | 12 | 36.4 | 6 | 22.2 | 18 | 46.2 |

*No tracheal wash sample collected on 5 occasions

A total of 314 tracheal wash and 319 nasopharyngeal swab samples were collected from 29 Welsh Mountain pony foals sampled on 11 separate occasions (10 consecutive weeks [weeks 1 - 10] and a further sampling 16 weeks later [week 26]).

*S. zooepidemicus* was isolated more frequently from tracheal washes than nasopharyngeal swabs, with 93.6% of washes and 87.5% of swabs being positive (*S.*

*zooepidemicus* prevalence). Of the 573 *S. zooepidemicus* isolates that were stored frozen after bacterial culture of samples, 538 (94%) were suitable and/or available for typing by characterisation of the 16S-23S RNA gene intergenic spacer and M-like protein hypervariable region using polymerase chain reaction (PCR).

PCR tests identified 39 different specific types of *S. zooepidemicus* among all isolates, with 33 types represented among tracheal wash isolates and 27 types among nasopharyngeal swab isolates. There were 21 specific types common to both tracheal wash and nasopharyngeal swab isolates and these represented 96% of all isolates. Of all isolates from washes, swabs and combined, 84% were represented by the 10 most prevalent specific types (Table 12.2, Figure 12.1).

**Table 12.2: Summary of specific *S. zooepidemicus* types isolated from tracheal wash (TW) and nasopharyngeal (NP) swab samples**

| *S. zooepidemicus* type | Isolates from TWs | | | Isolates from NP swabs | | |
|---|---|---|---|---|---|---|
| | n | % | cum. % | n | % | cum. % |
| A1 HV1 | 47 | 17.1 | 17.1 | 46 | 17.5 | 17.5 |
| A1 HV untyped | 39 | 14.2 | 31.3 | 41 | 15.6 | 33.1 |
| A1 HV3 | 28 | 10.2 | 41.5 | 20 | 7.6 | 40.7 |
| C1 HV3 | 26 | 9.5 | 50.9 | 31 | 11.8 | 52.5 |
| A1 HV4 | 18 | 6.5 | 57.5 | 18 | 6.8 | 59.3 |
| A1 HV2/3 | 16 | 5.8 | 63.3 | 13 | 4.9 | 64.3 |
| C1 HV2/3 | 16 | 5.8 | 69.1 | 17 | 6.5 | 70.7 |
| A1 HV2 | 14 | 5.1 | 74.2 | 13 | 4.9 | 75.7 |
| A1 HV2/4 | 14 | 5.1 | 79.3 | 11 | 4.2 | 79.8 |
| B1/D1 HV1 | 12 | 4.4 | 83.6 | 12 | 4.6 | 84.4 |
| Other types (Table 12.3) | 45 | 16.4 | 100 | 41 | 15.6 | 100 |
| Total | 275 | 100 | | 263 | 100 | |

Table 12.2 summarises the number, proportion and cumulative proportion of different specific *S. zooepidemicus* types isolated from tracheal washes and nasopharyngeal swabs. The proportions of all isolates that were specific types were very similar for both kinds of samples. Type A1 HV1 was the most prevalent type (>17%) isolated from both tracheal wash and nasopharyngeal swabs samples and for both these kinds of samples >50%

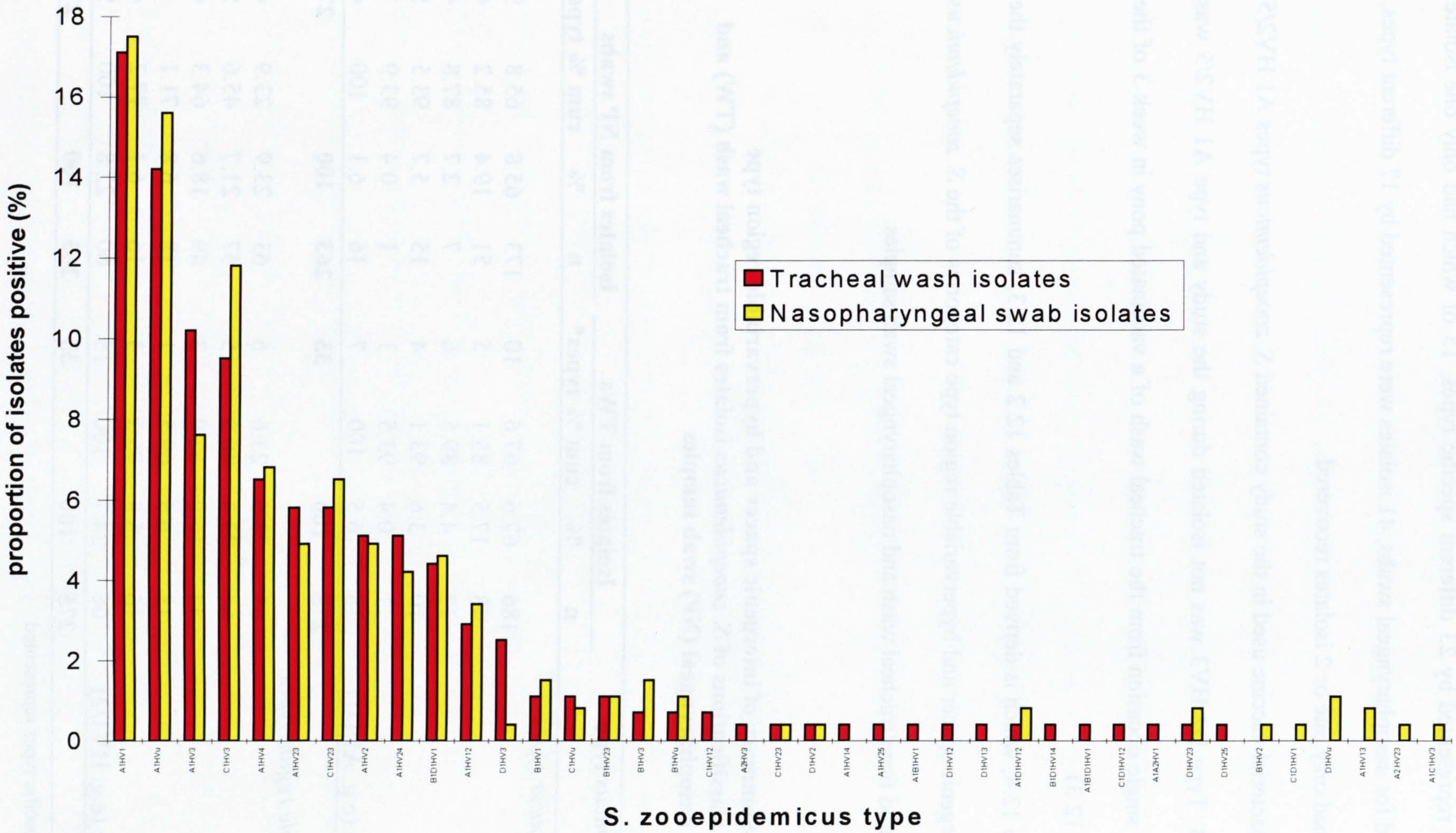
of isolates were represented by the 4 most prevalent types (A1 HV1, A1 HV untyped, A1 HV3 and C1 HV3).

**Table 12.3: Summary of numbers of the least prevalent *S. zooepidemicus* types isolated from tracheal wash (TW) and nasopharyngeal (NP) swab samples**

| *S. zooepidemicus* type | Isolates from TWs (n) | Isolates from NP swabs (n) | Isolates from TWs and/or NP swabs (n) |
|---|---|---|---|
| A1 HV1/2 | 8 | 9 | 17 |
| D1 HV3 | 7 | 1 | 8 |
| B1 HV1 | 3 | 4 | 7 |
| C1 HV untyped | 3 | 2 | 5 |
| B1 HV2/3 | 3 | 3 | 6 |
| B1 HV3 | 2 | 4 | 6 |
| B1 HV untyped | 2 | 3 | 5 |
| C1 HV1/2 | 2 | 0 | 2 |
| A2 HV3 | 1 | 0 | 1 |
| C1 HV2 | 1 | 1 | 2 |
| D1 HV2 | 1 | 1 | 2 |
| A1 HV1/4 | 1 | 0 | 1 |
| A1 HV2/5* | 1 | 0 | 1 |
| A1/B1 HV1 | 1 | 0 | 1 |
| D1 HV1/2 | 1 | 0 | 1 |
| D1 HV1/3 | 1 | 0 | 1 |
| A1/D1 HV1/2 | 1 | 2 | 3 |
| B1/D1 HV1/4 | 1 | 0 | 1 |
| A1/B1/D1 HV1 | 1 | 0 | 1 |
| C1/D1 HV1/2 | 1 | 0 | 1 |
| A1/A2 HV1 | 1 | 0 | 1 |
| D1 HV2/3 | 1 | 2 | 3 |
| D1 HV2/5 | 1 | 0 | 1 |
| B1 HV2 | 0 | 1 | 1 |
| C1/D1 HV1 | 0 | 1 | 1 |
| D1 HV untyped | 0 | 3 | 3 |
| A1 HV1/3 | 0 | 2 | 2 |
| A2 HV2/3 | 0 | 1 | 1 |
| A1/C1 HV3 | 0 | 1 | 1 |
| Total number of isolates | 45 | 41 | 86 |
| Total number of types | 23 | 17 | 29 |

* A1 HV2/5 was one of 2 types contained in the bacterial vaccine administered to the vaccine group

**Figure 12.1: Proportion of isolates positive in tracheal and nasopharyngeal samples for each of 39 specific *S. zooepidemicus* types**



Details of the specific *S. zooepidemicus* types represented by those other than the 10 most prevalent types are summarised in Table 12.3 and Figure 12.1. For tracheal washes, 45 isolates were represented by 23 different specific types, 15 of which had only one isolate recovered and for nasopharyngeal swabs, 41 isolates were represented by 17 different types, 11 of which had only one or 2 isolates recovered.

The bacterial vaccine used in the study contained *S. zooepidemicus* types A1 HV2/5 and D2 HV3. Type D2 HV3 was not isolated during the study and type A1 HV2/5 was isolated on a single occasion from the tracheal wash of a vaccinated pony in week 3 of the study (Table 12.3).

Table 12.4, which is derived from Tables 12.2 and 12.3, summarises separately the different intergenic spacer and hypervariable region type categories of the *S. zooepidemicus* isolates cultured from tracheal wash and nasopharyngeal swab samples.

**Table 12.4: Summary of intergenic spacer and hypervariable region type classifications of *S. zooepidemicus* isolates from tracheal wash (TW) and nasopharyngeal (NP) swab samples**

| *S. zooepidemicus* type | Isolates from TWs | | | | Isolates from NP swabs | | | |
|---|---|---|---|---|---|---|---|---|
| | n | % | cum % | types* | n | % | cum % | types* |
| *Intergenic spacer types* | | | | | | | | |
| A1 | 186 | 67.6 | 67.6 | 10 | 173 | 65.8 | 65.8 | 9 |
| C1 | 48 | 17.5 | 85.1 | 5 | 51 | 19.4 | 85.2 | 4 |
| D1 | 12 | 4.4 | 89.5 | 6 | 7 | 2.7 | 87.8 | 4 |
| B1 | 10 | 3.6 | 93.1 | 4 | 15 | 5.7 | 93.5 | 5 |
| A2 | 1 | 0.4 | 93.5 | 1 | 1 | 0.4 | 93.9 | 1 |
| Polymorphic (e.g. A1/D1) | 18 | 6.5 | 100 | 7 | 16 | 6.1 | 100 | 4 |
| **Total** | **275** | **100** | | **33** | **263** | **100** | | **27** |
| *Hypervariable region types* | | | | | | | | |
| HV1 | 65 | 23.6 | 23.6 | 6 | 63 | 23.9 | 23.9 | 4 |
| HV3 | 64 | 23.3 | 46.9 | 5 | 57 | 21.7 | 45.6 | 5 |
| HV untyped | 44 | 16.0 | 62.9 | 3 | 49 | 18.6 | 64.3 | 4 |
| HV4 | 18 | 6.5 | 69.5 | 1 | 18 | 6.8 | 71.1 | 1 |
| HV2 | 16 | 5.8 | 75.3 | 3 | 16 | 6.1 | 77.2 | 4 |
| Polymorphic (e.g. HV1/2) | 68 | 24.7 | 100 | 15 | 60 | 22.8 | 100 | 9 |
| **Total** | **275** | **100** | | **33** | **263** | **100** | | **27** |

*Number of specific types represented

Of the intergenic spacer types, type A1 was by far the most prevalent single type, accounting for approximately two-thirds of all isolates. Only 2 A2 types were isolated, each on a single occasion from a tracheal wash and nasopharyngeal swab. Although several specific intergenic spacer types, including C2, B2 and D2, were not among the isolates from the study, other polymorphic intergenic spacer types (i.e. those containing more than one type) were identified and these accounted for approximately 6% of all isolates.

Among the hypervariable region types, types HV1 and HV3 each accounted for a little under a quarter of all isolates. There were no HV5 types isolated and a much larger proportion (24%) of all isolates were represented by polymorphic hypervariable region types compared to those characterised by polymorphic intergenic spacer types (6%).

### 12.1.2 The 4 most prevalent *S. zooepidemicus* types

#### *12.1.2.1 Distribution of types over time*

Among both tracheal wash and nasopharyngeal swab samples, *S. zooepidemicus* types A1 HV1, A1 HV untyped (A1 HVu), A1 HV3 and C1 HV3 were the 4 most prevalent types, accounting for more than half of isolates for each sample category.

Figure 12.2 illustrates on separate graphs the proportion of ponies that were positive in tracheal wash and nasopharyngeal swab samples for each of the 4 most prevalent *S. zooepidemicus* types for the 10 consecutive weeks of sampling of the study. Results indicate that for each *S. zooepidemicus* type there was a broadly equivalent pattern of prevalence over time for both the tracheal and nasopharyngeal isolates. The prevalence pattern over time did, however, vary for individual *S. zooepidemicus* types and this is illustrated in Figure 12.3 which shows on 2 separate graphs the distribution of proportions of ponies with tracheal washes positive for 2 different types with equivalent peak prevalences. These results show that some *S. zooepidemicus* types such as A1 HV1 and C1 HV3 were relatively common in the earlier weeks after sampling started and then declined in the later

weeks. In contrast other types such as A1 HVu and A1 HV2/3 (polymorphic hypervariable region) demonstrated the reverse pattern of prevalence, only becoming more prevalent in the later weeks of sampling.

**Figure 12.2: Proportion of ponies positive by week for tracheal wash (TW) and nasopharyngeal (NP) isolates of the 4 most prevalent *S. zooepidemicus* types**

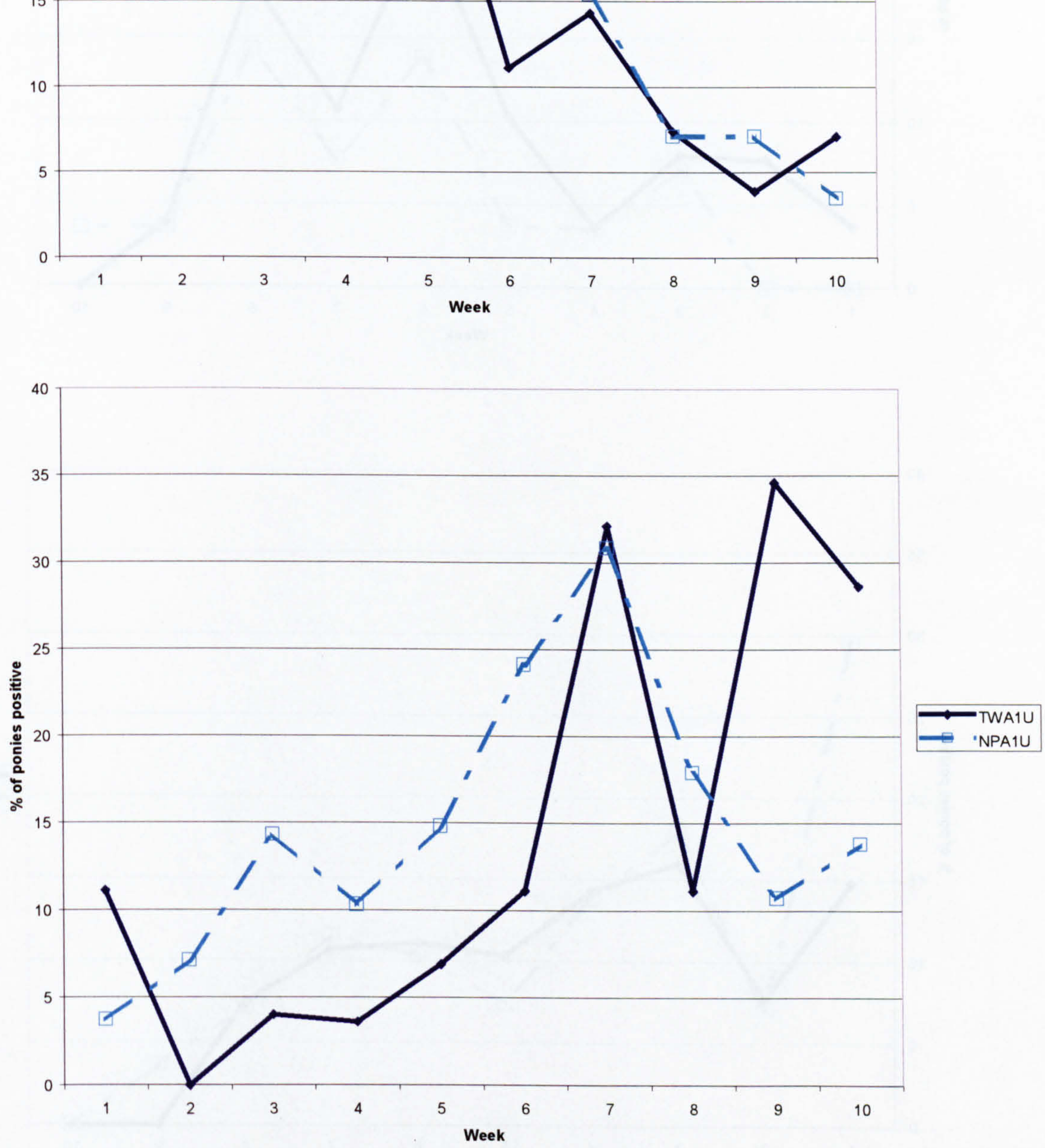


Figure 12.2 continued

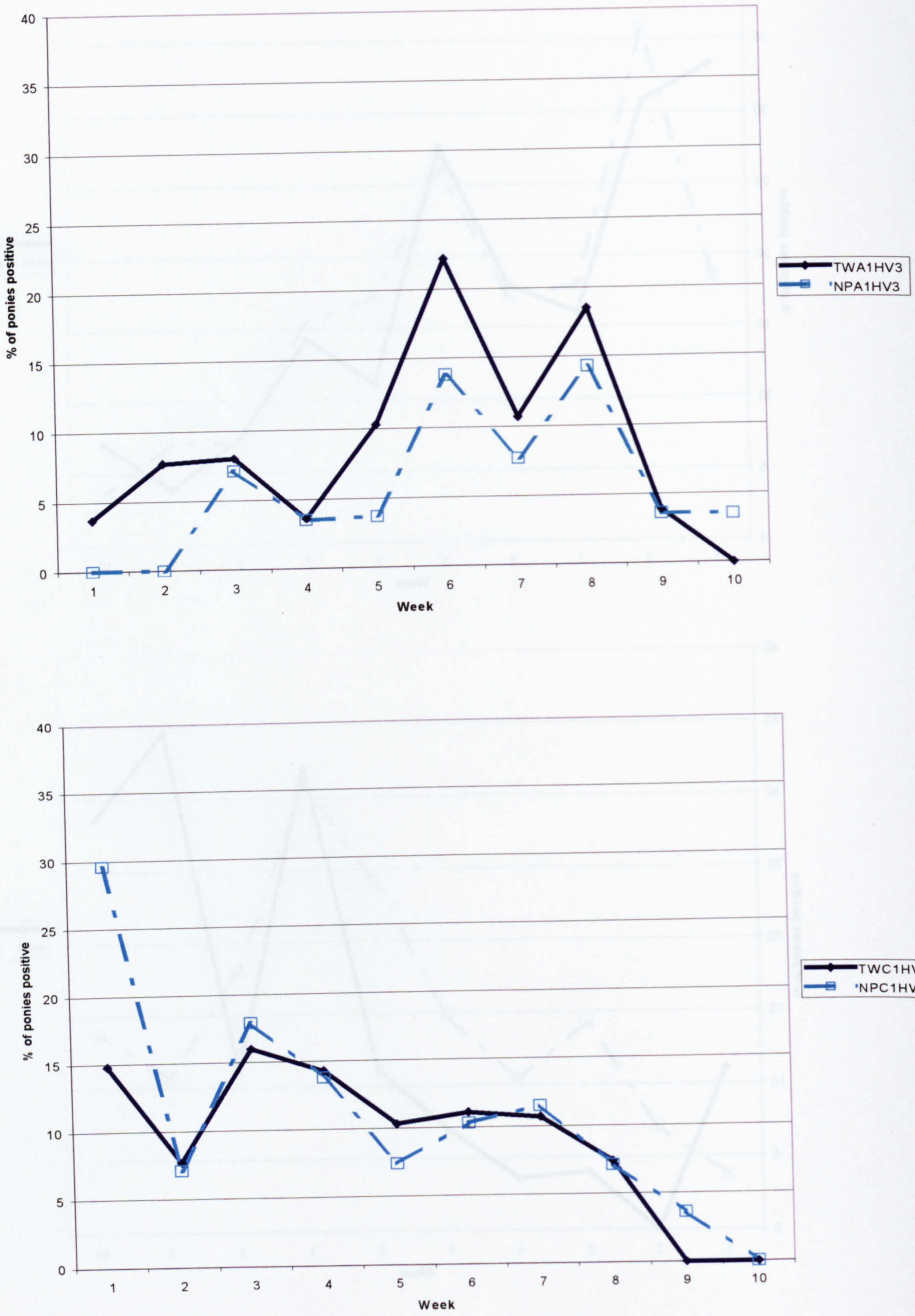
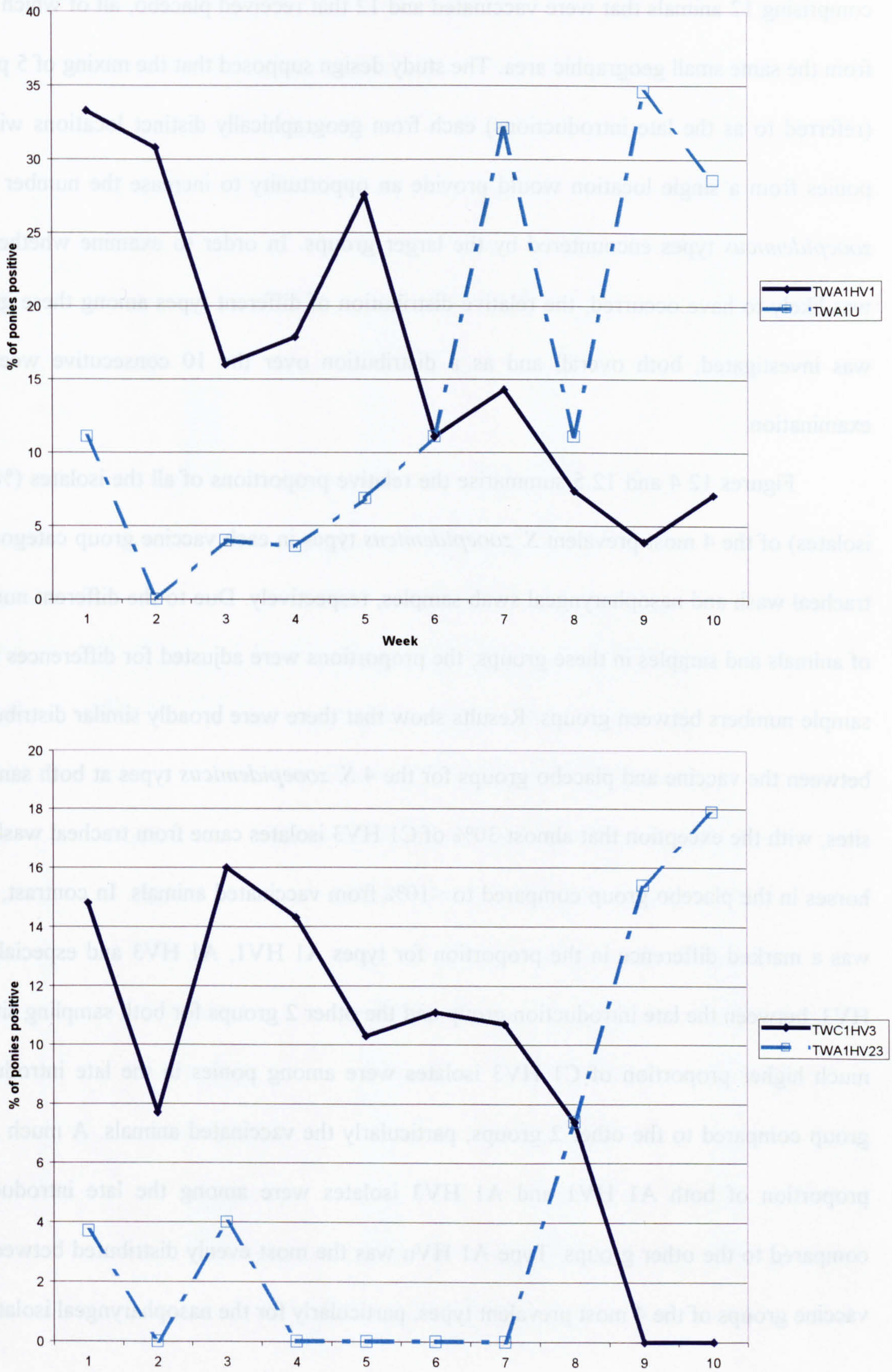


**Figure 12.3: Proportion of ponies positive by week for tracheal wash (TW) of different *S. zooepidemicus* types demonstrates distinct variation in temporal distribution**



### *12.1.2.2 Distribution of types among vaccine groups and over time*

The 29 ponies in the study were allocated to different vaccine groups. These included 5 non-vaccine or placebo ponies from 5 different locations in Wales and 24 ponies, comprising 12 animals that were vaccinated and 12 that received placebo, all of which were from the same small geographic area. The study design supposed that the mixing of 5 ponies (referred to as the late introductions) each from geographically distinct locations with 24 ponies from a single location would provide an opportunity to increase the number of *S. zooepidemicus* types encountered by the larger groups. In order to examine whether this was likely to have occurred, the relative distribution of different types among these groups was investigated, both overall and as a distribution over the 10 consecutive weeks of examination.

Figures 12.4 and 12.5 summarise the relative proportions of all the isolates (% of n isolates) of the 4 most prevalent *S. zooepidemicus* types in each vaccine group category for tracheal wash and nasopharyngeal swab samples, respectively. Due to the different numbers of animals and samples in these groups, the proportions were adjusted for differences in the sample numbers between groups. Results show that there were broadly similar distributions between the vaccine and placebo groups for the 4 *S. zooepidemicus* types at both sampling sites, with the exception that almost 30% of C1 HV3 isolates came from tracheal washes of horses in the placebo group compared to <10% from vaccinated animals. In contrast, there was a marked difference in the proportion for types A1 HV1, A1 HV3 and especially C1 HV3, between the late introduction group and the other 2 groups for both sampling sites. A much higher proportion of C1 HV3 isolates were among ponies in the late introduction group compared to the other 2 groups, particularly the vaccinated animals. A much lower proportion of both A1 HV1 and A1 HV3 isolates were among the late introductions compared to the other groups. Type A1 HVu was the most evenly distributed between the vaccine groups of the 4 most prevalent types, particularly for the nasopharyngeal isolates.

**Figure 12.4: Distribution of tracheal wash isolates of the 4 most prevalent *S. zooepidemicus* types by vaccine group, adjusting for differences in sample numbers**

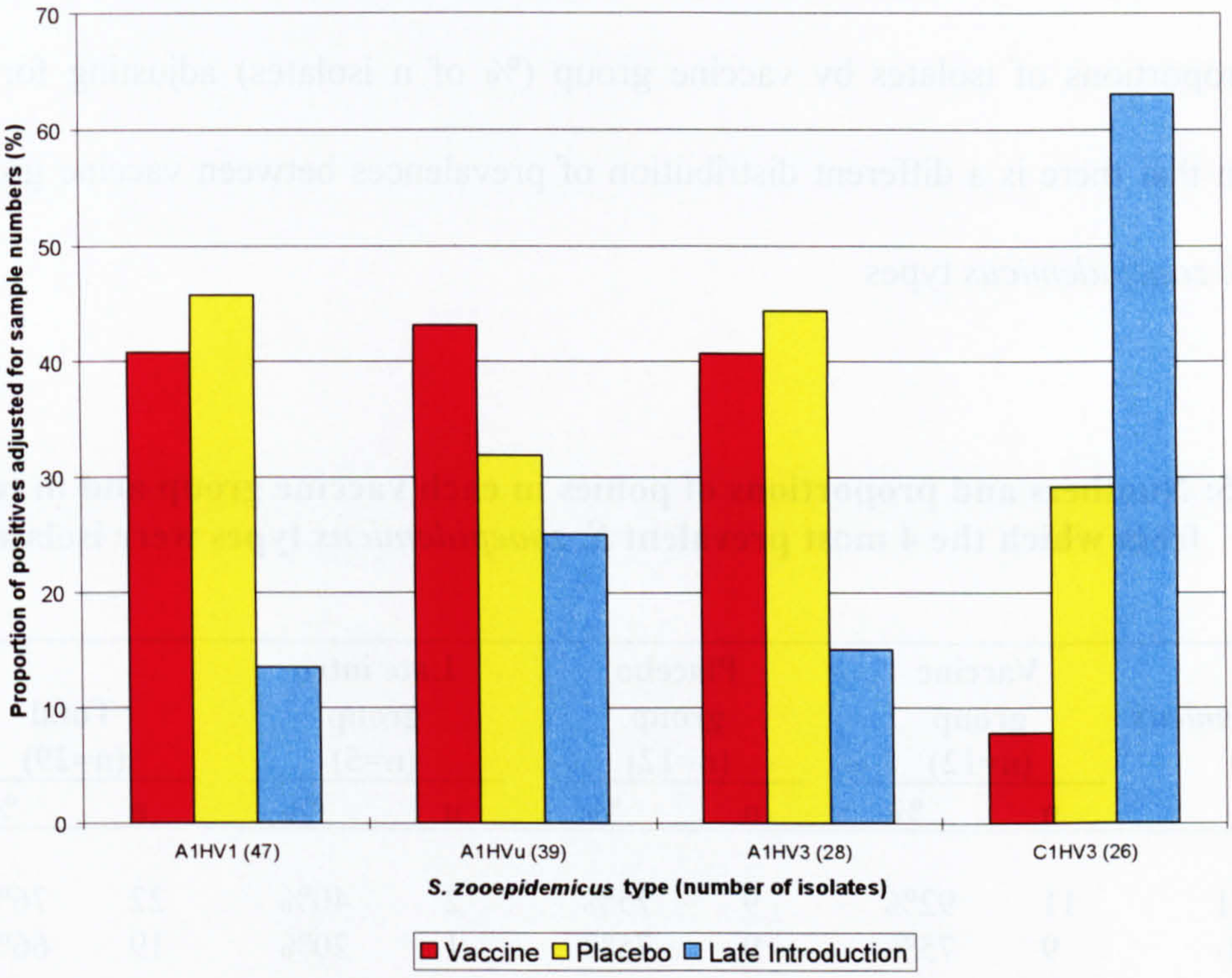



**Figure 12.5: Distribution of nasopharyngeal isolates of the 4 most prevalent *S. zooepidemicus* types by vaccine group, adjusting for differences in sample numbers**

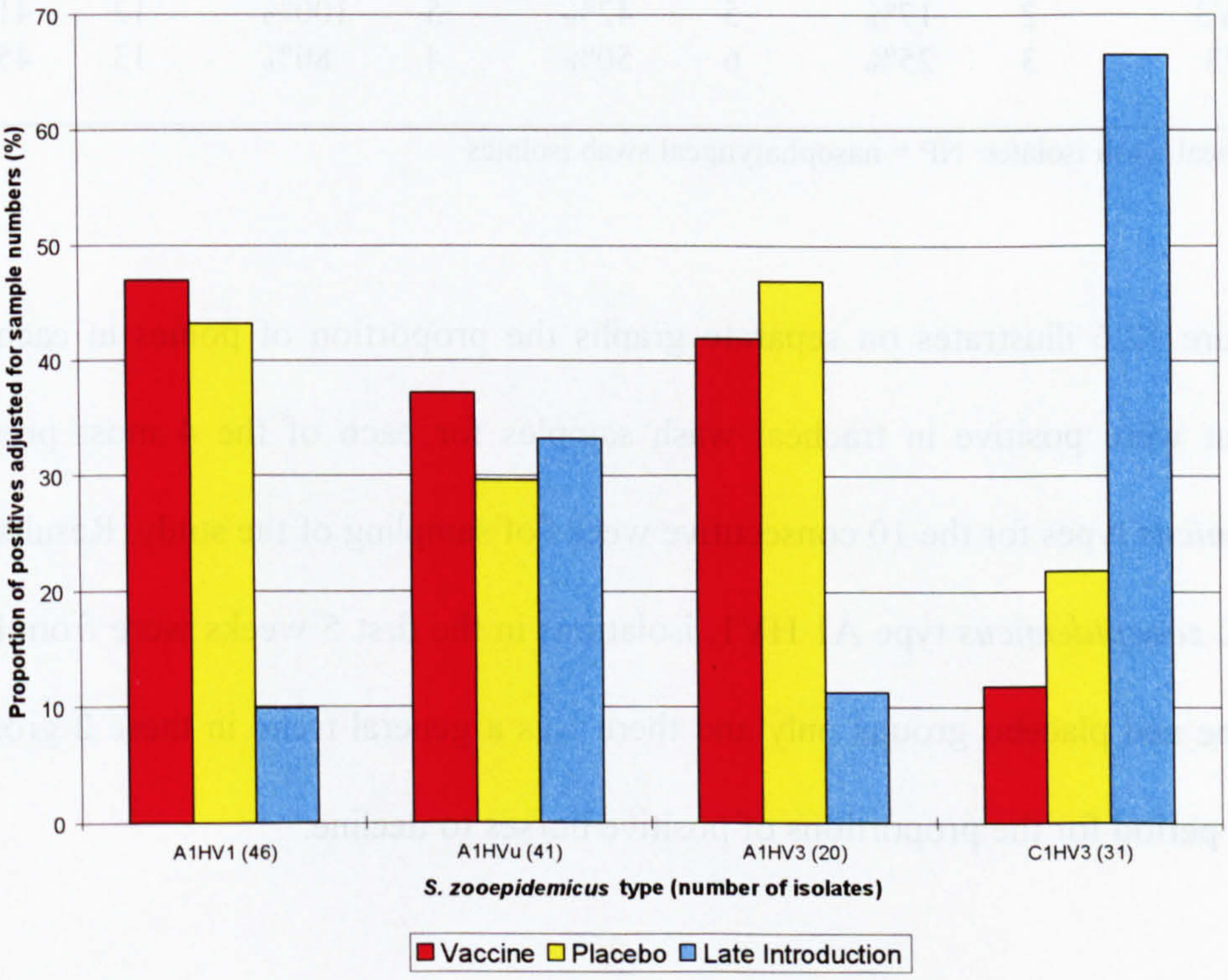


Table 12.5 summarises separately for tracheal and nasopharyngeal samples the total numbers and proportions of ponies in each vaccine group from which each of the 4 most prevalent *S. zooepidemicus* types were isolated. Results are broadly similar to those for the relative proportions of isolates by vaccine group (% of n isolates) adjusting for sample numbers in that there is a different distribution of prevalences between vaccine groups for different *S. zooepidemicus* types.

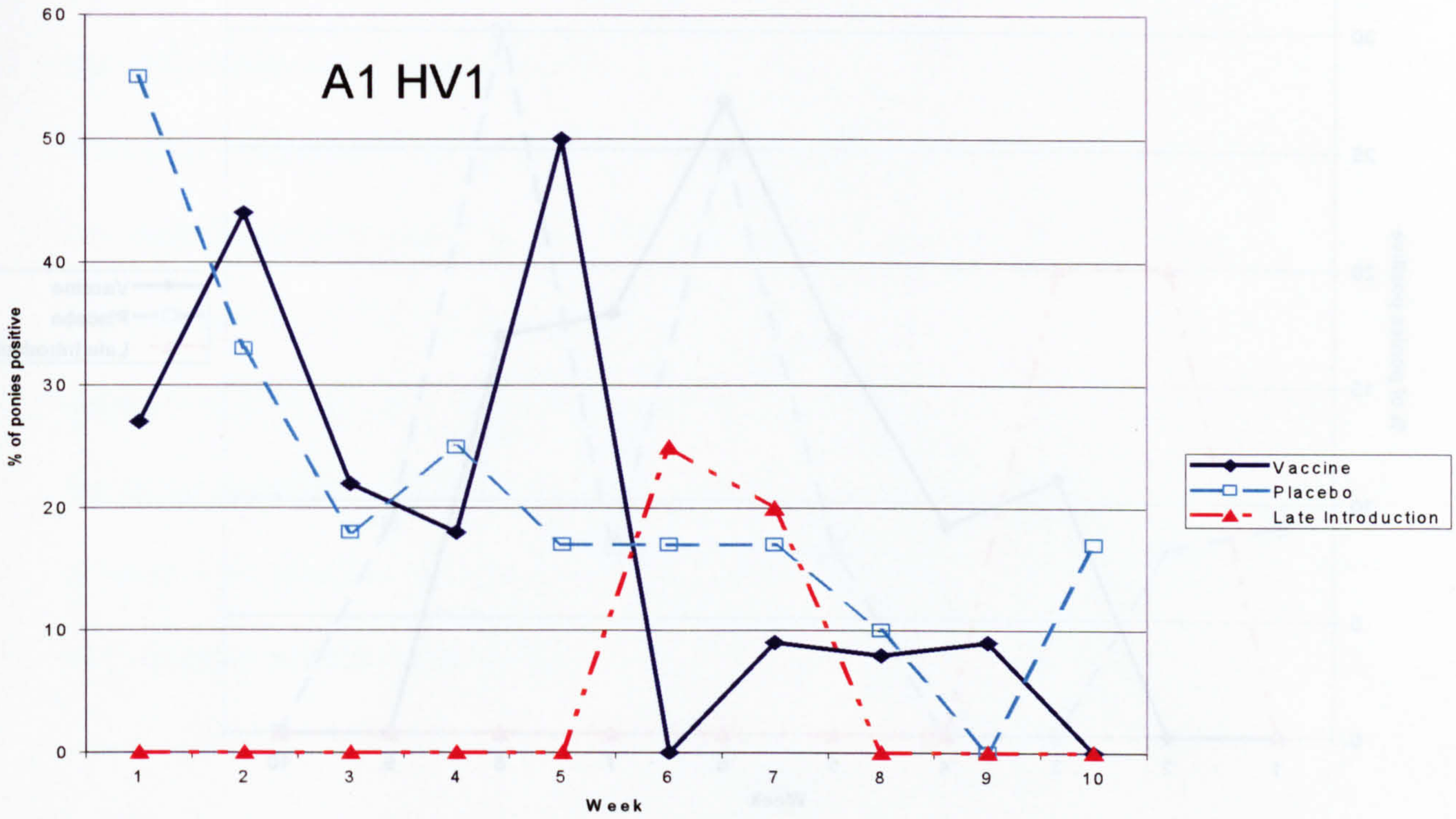
**Table 12.5: Numbers and proportions of ponies in each vaccine group and in total from which the 4 most prevalent *S. zooepidemicus* types were isolated**

| *S. zooepidemicus* type | Vaccine group (n=12) | | Placebo group (n=12) | | Late intro group (n=5) | | Total (n=29) | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % |
| TW A1HV1 | 11 | 92% | 9 | 75% | 2 | 40% | 22 | 76% |
| NP A1HV1 | 9 | 75% | 9 | 75% | 1 | 20% | 19 | 66% |
| TW A1HVu | 10 | 83% | 9 | 75% | 2 | 40% | 21 | 72% |
| NP A1HVu | 8 | 67% | 8 | 67% | 4 | 80% | 20 | 69% |
| TW A1HV3 | 6 | 50% | 9 | 75% | 1 | 20% | 16 | 55% |
| NP A1HV3 | 4 | 33% | 7 | 58% | 1 | 20% | 12 | 41% |
| TW C1HV3 | 2 | 17% | 5 | 42% | 5 | 100% | 12 | 41% |
| NP C1HV3 | 3 | 25% | 6 | 50% | 4 | 80% | 13 | 45% |

TW = tracheal wash isolates NP = nasopharyngeal swab isolates

Figure 12.6 illustrates on separate graphs the proportion of ponies in each vaccine group that were positive in tracheal wash samples for each of the 4 most prevalent *S. zooepidemicus* types for the 10 consecutive weeks of sampling of the study. Results indicate that for *S. zooepidemicus* type A1 HV1, isolations in the first 5 weeks were from horses in the vaccine and placebo groups only and there was a general trend in these 2 groups over the entire period for the proportions of positive horses to decline.

**Figure 12.6: Proportion by week of ponies of each vaccine group positive for tracheal wash (TW) isolates of the 4 most prevalent *S. zooepidemicus* types**



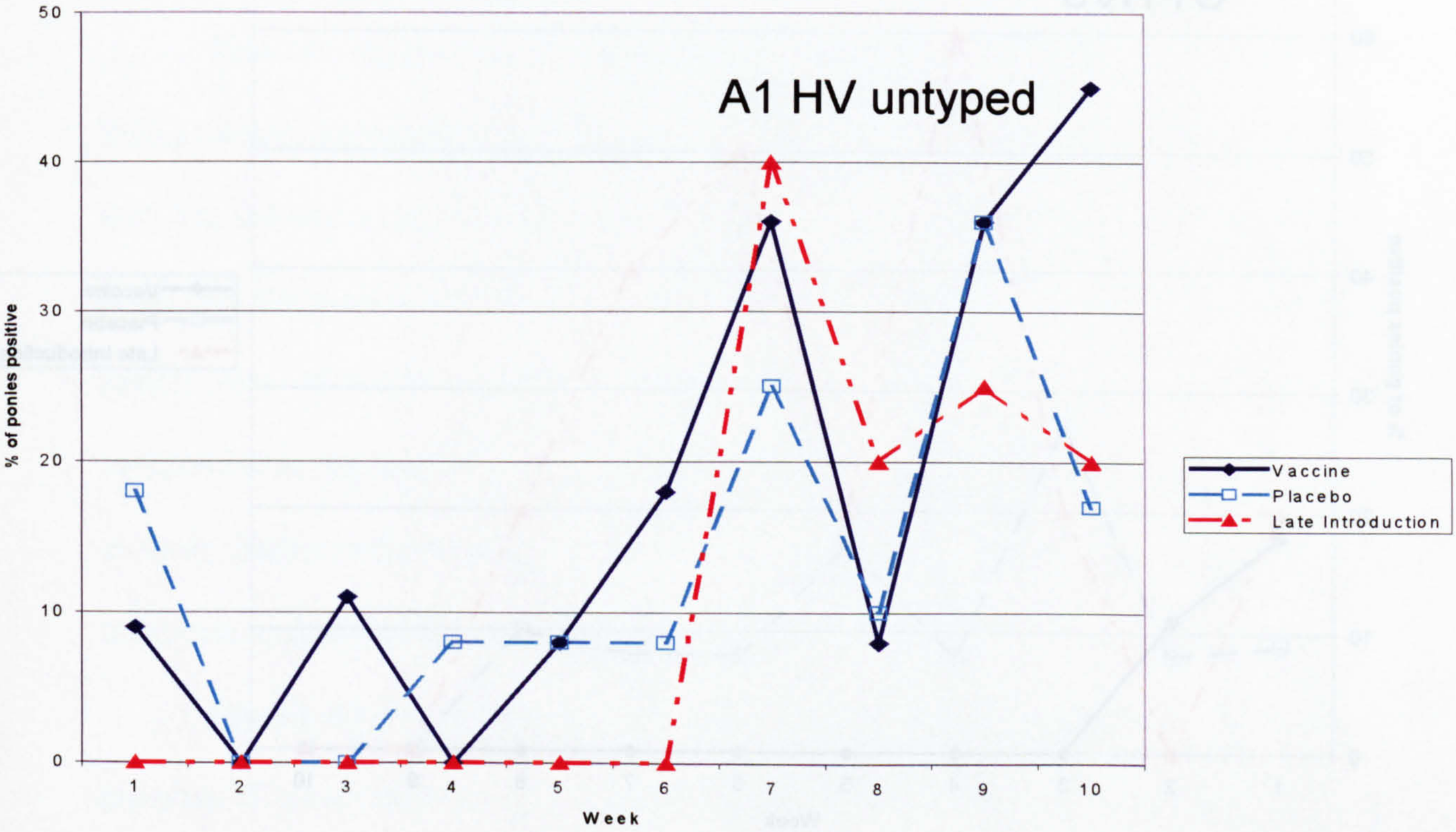
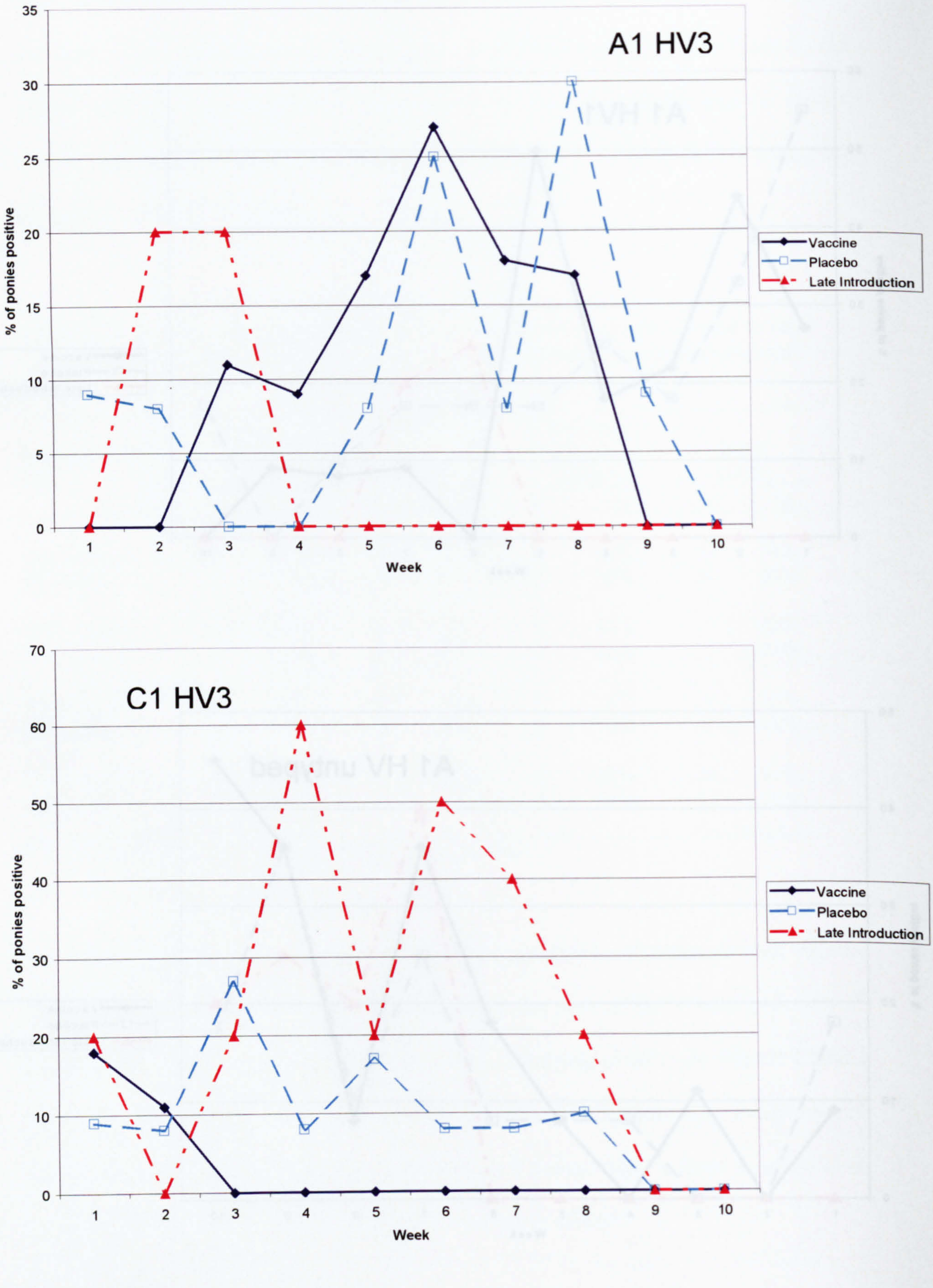



Figure 12.6 continued



In weeks 6 and 7 of the study only one pony from the late introduction group was positive in tracheal washes for type A1 HV1. For type A1 HVu there was a similar distribution over time for all 3 groups, with the proportion of positive animals increasing markedly in the last 4 weeks compared to the first 6 weeks. For type A1 HV3 there was a difference in temporal distribution of proportion of positive ponies by week between the late introduction and the other 2 groups. The peak for the late introduction group occurred in weeks 2 and 3 but for the other groups was later at weeks 6 to 8. For type C1 HV3 the small proportion of positive vaccine administered ponies occurred in the first 2 weeks, for the larger proportion of placebo administered ponies the peak proportion infected occurred at week 3 and declined to zero in week 9 and for the late introduction group most animals were infected between weeks 5 and 7.

## 12.2 Association of *S. zooepidemicus* types with respiratory disease

### 12.2.1 Aggregated clinical sign and airway inflammation scores

#### *12.2.1.1 Univariable analyses*

Table A3.1 (Appendix 3) summarises the results and comparisons of linear and best fitting polynomial regression analyses of clinical, CDNS and airway inflammation scores with $\log_{10}$ cfu/ml tracheal wash counts for each of the 5 most prevalent *S. zooepidemicus* types (A1 HV1, A1 HVu, A1 HV3, C1 HV3 and A1 HV4). All analyses were based on a sample size of n=295 which comprised 275 sampling occasions when tracheal *S. zooepidemicus* isolates were typed and 20 occasions when *S. zooepidemicus* was not isolated. Higher order models containing 3 or more power terms were investigated but found not to significantly improve the fit of the data.

Figures A3.1 - A3.3, each comprising 5 component graphs for each of the 5 most prevalent *S. zooepidemicus* types, represented the individual data points of $\log_{10}$ cfu/ml in tracheal washes plotted against the corresponding clinical (Figure A3.1), CDNS (Figure

A3.2) and airway inflammation (Figure A3.3) scores and include the summary regression line of the best fitting polynomial regression.

Comparison of the respective linear and polynomial model $R^2$ values showed that in all cases the fitting of a 2-power polynomial regression model increased the amount of variability in the data explained by the models. The last 2 columns of Table A3.1 represented the decrease in deviance by the polynomial model compared to the linear regression and the corresponding P-value based on 3 degrees of freedom in a chi-squared distribution. This indicated whether the polynomial model provided a significantly improved fit of the data compared to the linear model.

Results showed a similar pattern across each of the 3 outcome measures, in that the same *S. zooepidemicus* types generally demonstrated similar relationships between the linear and polynomial regressions. Linear regression of outcome scores with $log_{10}$ cfu/ml of types A1 HV1 and A1 HV3 did not demonstrate a significant association between bacterial numbers and increasing outcome scores, but polynomial regression predicted a significant, non-linear relationship (Figures A3.1-A3.3). This was also true for $log_{10}$ cfu/ml of C1 HV3 with airway inflammation score. $Log_{10}$ cfu/ml of types A1 HVu and C1 HV3 showed significant positive association with clinical and CDNS scores by both linear and polynomial regressions and although there was no evidence of a significant difference between the 2, $R^2$ values were greater in the polynomial models. There was a similar relationship between the polynomial and linear regression models for $log_{10}$ cfu/ml of A1 HVu and A1 HV4 and airway inflammation score. Although linear regression of A1 HV4 with clinical and CDNS scores was significant there was also significant improvement and increased $R^2$s with polynomial regression.

Table 12.6 summarises the results of non-parametric analyses (Wilcoxon rank sum test) for significant differences in clinical, CDNS and airway inflammation scores with the isolation of each of the 5 most prevalent *S. zooepidemicus* types on nasopharyngeal swabs.

Results showed that although there was no statistically significant association at P<0.05 between clinical and CDNS scores and whether specific types of *S. zooepidemicus* were isolated from nasopharyngeal swabs, there was evidence that CDNS scores were higher when types C1 HV3 or A1 HV4 were isolated from swabs (P<0.07). Consistent with this, airway inflammation scores were significantly higher when types C1 HV3 or A1 HV4 were isolated from swabs.

**Table 12.6: Summary of non-parametric analyses examining differences in clinical, CDNS & airway inflammation scores with isolation of the 5 most prevalent *S. zooepidemicus* types on nasopharyngeal swabs**

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Clinical score** | Nasopharyngeal A1 HV1 isolate | Wilcoxon rank sum | 0.510 |
| | Nasopharyngeal A1 HVu isolate | Wilcoxon rank sum | 0.377 |
| | Nasopharyngeal A1 HV3 isolate | Wilcoxon rank sum | 0.519 |
| | Nasopharyngeal C1 HV3 isolate | Wilcoxon rank sum | 0.176 |
| | Nasopharyngeal A1 HV4 isolate | Wilcoxon rank sum | 0.073 |
| **CDNS score** | Nasopharyngeal A1 HV1 isolate | Wilcoxon rank sum | 0.466 |
| | Nasopharyngeal A1 HVu isolate | Wilcoxon rank sum | 0.384 |
| | Nasopharyngeal A1 HV3 isolate | Wilcoxon rank sum | 0.839 |
| | **Nasopharyngeal C1 HV3 isolate** | **Wilcoxon rank sum** | **0.068** |
| | **Nasopharyngeal A1 HV4 isolate** | **Wilcoxon rank sum** | **0.061** |
| **Airway inflammation score** | Nasopharyngeal A1 HV1 isolate | Wilcoxon rank sum | 0.091 |
| | Nasopharyngeal A1 HVu isolate | Wilcoxon rank sum | 0.634 |
| | Nasopharyngeal A1 HV3 isolate | Wilcoxon rank sum | 0.344 |
| | **Nasopharyngeal C1 HV3 isolate** | **Wilcoxon rank sum** | **0.018** |
| | **Nasopharyngeal A1 HV4 isolate** | **Wilcoxon rank sum** | **0.042** |

### *12.2.1.2 Multivariable analyses excluding pony-level random effects*

Table 12.7 summarises the results from the final ordinary multiple linear regression analysis for the clinical score outcome variable including different *S. zooepidemicus* types as explanatory variables. As previously, 2 final models are presented, corresponding to separate models for the inclusion of transferrin D and transferrin F2 haplotypes. Both models are virtually identical other than for the direction of the regression coefficient

estimate for the transferrin haplotype terms. Final models included 2 autoregressive variables for the clinical score the week and 2 weeks previously, which had the effect of reducing the sample size to n=218.

**Table 12.7: Results of multivariable linear regression of clinical score, including individual terms for *S. zooepidemicus* types**

| Outcome variable / Model | Explanatory variables in the models (n) | Regression coefficient | 95% CI of coefficient | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|
| **Clinical score** | (n=218) | | | | |
| Model 1 | Intercept | 6.88 | 3.34 – 10.4 | 57.3 | <0.001 |
| | Clinical score 1 week previously | 0.42 | 0.29 – 0.55 | | <0.001 |
| | Clinical score 2 weeks previously | 0.15 | 0.03 – 0.27 | | 0.016 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.49 | -0.84 – -0.14 | | 0.006 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.07 | 0.02 – 0.13 | | 0.010 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.39 | -0.68 – -0.10 | | 0.008 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.07 | 0.02 – 0.12 | | 0.008 |
| | $Log_{10}$ cfu/ml A1 HVu | -1.04 | -2.11 – 0.03 | | 0.056 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.56 | 0.11 – 1.01 | | 0.016 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.06 | -0.11 – -0.01 | | 0.010 |
| | $Log_{10}$ cfu/ml A1 HV3 | -0.39 | -0.89 – 0.12 | | 0.134 |
| | $[Log_{10}$ cfu/ml A1 HV3$]^2$ | 0.10 | 0.01 – 0.19 | | 0.038 |
| | $Log_{10}$ cfu/ml C1 HV3 | 0.23 | 0.09 – 0.37 | | 0.001 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-1}$ | 0.92 | 0.25 – 1.59 | | 0.007 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | -9.70 | -16.8 – -2.64 | | 0.007 |
| | Transferrin D haplotype | -0.47 | -0.87 – -0.07 | | 0.021 |
| Model 2 | Intercept | 5.88 | 2.36 – 9.41 | 57.0 | 0.001 |
| | Clinical score 1 week previously | 0.43 | 0.30 – 0.56 | | <0.001 |
| | Clinical score 2 weeks previously | 0.14 | 0.02 – 0.26 | | 0.021 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.46 | -0.81 – -0.11 | | 0.010 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.08 | 0.02 – 0.13 | | 0.008 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.41 | -0.70 – -0.12 | | 0.005 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.07 | 0.02 – 0.12 | | 0.007 |
| | $Log_{10}$ cfu/ml A1 HVu | -1.08 | -2.15 – -0.01 | | 0.048 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.57 | 0.12 – 1.03 | | 0.014 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.06 | -0.11 – -0.02 | | 0.009 |
| | $Log_{10}$ cfu/ml A1 HV3 | -0.43 | -0.93 – 0.08 | | 0.100 |
| | $[Log_{10}$ cfu/ml A1 HV3$]^2$ | 0.10 | 0.01 – 0.19 | | 0.031 |
| | $Log_{10}$ cfu/ml C1 HV3 | 0.22 | 0.08 – 0.36 | | 0.003 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-1}$ | 0.85 | 0.17 – 1.53 | | 0.014 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | -8.95 | -16.1 – -1.85 | | 0.014 |
| | Transferrin F2 haplotype | 0.42 | -0.01 – 0.85 | | 0.054 |

Models also included various terms for $log_{10}$ cfu/ml counts of tracheal wash bacteria including different *S. zooepidemicus* types. Retained significant terms included counts of non-haemolytic S*treptococcus* spp., *Pasteurella* spp. and A1 HV3 type *S. zooepidemicus* all expressed as quadratic terms. *S. zooepidemicus* type A1 HVu was expressed as a cubic

term, C1 HV3 as a linear term and A1 HV4 as a polynomial term. It is of note that the most prevalent *S. zooepidemicus* type, A1 HV1, was not statistically associated with clinical score in whatever form it was modelled. The $R^2$ values for these models indicated that 57% of the variability of clinical score was explained by the factors retained in them.

Table 12.8 summarises the results from the final ordinary multiple linear regression analyses for the CDNS and airway inflammation score outcome variables, including different *S. zooepidemicus* types as explanatory variables. As for clinical score, 2 final models are presented for CDNS score, corresponding to models containing variables for different transferrin haplotypes. Both CDNS score models were virtually identical to those for clinical score with the exception that only one autoregressive outcome variable (CDNS score one week previously) was retained, this increased the available sample size to n=244.

The final model for airway inflammation score also included several terms for $\log_{10}$ cfu/ml counts of tracheal wash bacteria including different *S. zooepidemicus* types. Retained significant or approaching significant terms included counts of non-haemolytic *Streptococcus* spp expressed as a quadratic, *S. zooepidemicus* types A1 HVu and C1 HV3 expressed as polynomials and A1 HV4 as a linear term. Again the most prevalent *S. zooepidemicus* type, A1 HV1, was not statistically associated with airway inflammation score in whatever form it was modelled. The $R^2$ values for this model indicated that 40% of the variability of airway inflammation score was explained by the factors included in the model.

## Table 12.8: Results of multivariable linear regression of CDNS and airway inflammation (AI) score, including individual terms for *S. zooepidemicus* types

| Outcome variable / Model | Explanatory variables in the models (n) | Regression coefficient | 95% CI of coefficient | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|
| **CDNS score** | (n=244) | | | | |
| Model 1 | Intercept | 4.90 | 2.12 – 7.67 | 57.3 | 0.001 |
| | CDNS score 1 week previously | 0.51 | 0.40 – 0.61 | | <0.001 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.45 | -0.73 – -0.17 | | 0.002 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.06 | 0.02 – 0.10 | | 0.008 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.34 | -0.56 – -0.11 | | 0.003 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.06 | 0.02 – 0.10 | | 0.003 |
| | $Log_{10}$ cfu/ml A1 HVu | -0.71 | -0.60 – 0.18 | | 0.116 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.40 | 0.02 – 0.78 | | 0.037 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.04 | -0.08 – -0.004 | | 0.027 |
| | $Log_{10}$ cfu/ml A1 HV3 | -0.34 | -0.74 – 0.07 | | 0.104 |
| | $[Log_{10}$ cfu/ml A1 HV3$]^2$ | 0.07 | 0.001 – 0.01 | | 0.046 |
| | $Log_{10}$ cfu/ml C1 HV3 | 0.13 | 0.01 – 0.24 | | 0.027 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-2}$ | 0.006 | 0.001 – 0.10 | | 0.016 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | -6.50 | -11.8 – -1.21 | | 0.016 |
| | Transferrin D haplotype | -0.40 | -0.71 – -0.10 | | 0.010 |
| Model 2 | Intercept | 4.07 | 1.30 – 6.84 | 57.2 | 0.004 |
| | CDNS score 1 week previously | 0.51 | 0.40 – 0.61 | | <0.001 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.42 | -0.70 – -0.15 | | 0.003 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.06 | 0.02 – 0.11 | | 0.006 |
| | $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.35 | 0.58 – -0.13 | | 0.002 |
| | $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.06 | 0.02 – 0.10 | | 0.002 |
| | $Log_{10}$ cfu/ml A1 HVu | -0.72 | -1.61 – 0.16 | | 0.110 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.41 | 0.03 – 0.78 | | 0.036 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.04 | -0.08 – -0.004 | | 0.027 |
| | $Log_{10}$ cfu/ml A1 HV3 | -0.36 | -0.77 – 0.04 | | 0.080 |
| | $[Log_{10}$ cfu/ml A1 HV3$]^2$ | 0.08 | 0.004 – 0.15 | | 0.039 |
| | $Log_{10}$ cfu/ml C1 HV3 | 0.12 | 0.006 – 0.23 | | 0.039 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-2}$ | 0.01 | 0.001 – 0.01 | | 0.028 |
| | $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | -5.90 | -11.2 – -0.61 | | 0.029 |
| | Transferrin F2 haplotype | 0.42 | 0.10 – 0.74 | | 0.011 |
| **Airway inflammation score** | (n=214) | | | | |
| | Intercept | 3.65 | -0.20 – 7.50 | 39.6 | 0.063 |
| | AI score 1 week previously | 0.30 | 0.18 – 0.43 | | <0.001 |
| | AI score 2 weeks previously | 0.20 | 0.10 – 0.31 | | <0.001 |
| | $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.59 | -0.93 – -0.25 | | 0.001 |
| | $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.09 | 0.04 – 0.15 | | 0.002 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^{-1}$ | 0.40 | 0.12 – 0.69 | | 0.006 |
| | $[Log_{10}$ cfu/ml A1 HVu$]^{-0.5}$ | -4.29 | -7.34 – -1.24 | | 0.006 |
| | $[Log_{10}$ cfu/ml C1 HV3$]^{-0.5}$ | 1.11 | -0.07 – 2.30 | | 0.066 |
| | $Log_n [Log_{10}$ cfu/ml C1 HV3] | 1.83 | -0.03 – 3.69 | | 0.054 |
| | $Log_{10}$ cfu/ml A1 HV4 | 0.16 | -0.002 – 0.32 | | 0.053 |

### *12.2.1.3 Multivariable analyses accounting for pony-level random effects*

Table A3.2 summarises the results from multilevel linear regression modelling for the outcome variables of clinical score and CDNS score with inclusion of autoregressive

explanatory variables, different tracheal wash bacterial variables including *S. zooepidemicus* types and transferrin haplotype variables. Table A3.3 shows similar results for the outcome variable of airway inflammation score. Tables 12.9 and 12.10 summarise the final results from multilevel linear regression modelling for the 3 outcome variables.

Sequential inclusion of terms for the different *S. zooepidemicus* types and where relevant transferrin haplotypes, resulted in significant improvements of model fit as judged by reduction in deviance and significance of likelihood ratio statistics. Comparison of final multilevel models with corresponding final multivariable linear regression models (Tables 12.7 and 12.8) shows they were very similar in terms of the direction and value of estimated coefficients and this was consistent with the non-significant pony-level random effect terms in the final multilevel models for clinical and airway inflammation scores. The final multilevel models for CDNS score had evidence that the pony-level random effect terms were just statistically significant.

# Table 12.9: Summary of multilevel linear regression modelling of clinical and CDNS scores, including individual terms for *S. zooepidemicus* types

| **Outcome variable** / Effect type / Explanatory variable in model | **Estimate (S.E.)** | **Estimate (S.E.)** |
|---|---|---|
| **Clinical score** | **Model 5a n=218** | **Model 5b n=218** |
| Fixed effect | | |
| Intercept | 7.28 (1.73) | 6.26 (1.71) |
| Clinical score 1 week previously | 0.38 (0.06) | 0.38 (0.06) |
| Clinical score 2 weeks previously | 0.13 (0.06) | 0.12 (0.06) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.48 (0.17) | -0.46 (0.17) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.07 (0.03) | 0.08 (0.03) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.38 90.14) | -0.39 (0.14) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp$]^2$ | 0.06 (0.02) | 0.07 (0.02) |
| $Log_{10}$ cfu/ml A1 Hvu | -0.93 (0.52) | -0.90 (0.52) |
| $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.52 (0.22) | 0.50 (0.22) |
| $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.06 (0.02) | -0.05 (0.02) |
| $Log_{10}$ cfu/ml A1 HV3 | -0.35 (0.25) | -0.36 (0.24) |
| $[Log_{10}$ cfu/ml A1 HV3$]^2$ | 0.09 (0.04) | 0.09 (0.04) |
| $Log_{10}$ cfu/ml C1 HV3 | 0.22 (0.07) | 0.21 (0.07) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-1}$ | 0.98 (0.33) | 0.90 (0.33) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | -10.3 (3.45) | -9.48 (3.41) |
| Transferrin D phenotype | -0.53 (0.23) | |
| Transferrin F2 phenotype | | 0.52 (0.25) |
| Random effect | | |
| Level 2 variance: Pony | 0.09 (0.08) | 0.14 (0.09) |
| Level 1 variance: Observation | 1.54 (0.16) | 1.52 (0.16) |
| *-2*loglikelihood* | *723.16* | *724.28* |
| *LRS $\chi^2$ (d.f.)* | *5.36 (1)* | *4.24 (1)* |
| *P-value* | *0.021* | *0.040* |
| *Intra-pony correlation (%)* | *5.8* | *8.2* |
| **CDNS score** | **Model 5a n=244** | **Model 5b n=244** |
| Fixed effect | | |
| Intercept | 5.13 (1.33) | 4.42 (1.34) |
| CDNS score 1 week previously | 0.42 (0.05) | 0.42 (0.05) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.40 (0.14) | -0.38 (0.14) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.05 (0.02) | 0.05 (0.02) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.33 (0.11) | -0.34 (0.11) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp$]^2$ | 0.06 (0.02) | 0.06 (0.02) |
| $Log_{10}$ cfu/ml A1 Hvu | -0.59 (0.43) | -0.58 (0.42) |
| $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.34 (0.18) | 0.34 (0.18) |
| $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.04 (0.02) | -0.04 (0.02) |
| $Log_{10}$ cfu/ml A1 HV3 | -0.30 (0.19) | -0.31 (0.19) |
| $[Log_{10}$ cfu/ml A1 HV3$]^2$ | 0.07 (0.04) | 0.07 (0.04) |
| $Log_{10}$ cfu/ml C1 HV3 | 0.12 (0.06) | 0.11 (0.06) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-2}$ | 0.01 (0.002) | 0.01 (0.002) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-0\,5}$ | -6.86 (2.51) | -6.56 (2.52) |
| Transferrin D phenotype | -0.45 (0.21) | |
| Transferrin F2 phenotype | | 0.48 (0.21) |
| Random effect | | |
| Level 2 variance: Pony | 0.15 (0.07) | 0.15 (0.07) |
| Level 1 variance: Observation | 1.04 (0.10) | 1.03 (0.10) |
| *-2*loglikelihood* | *723.96* | *723.47* |
| *LRS $\chi^2$ (d.f.)* | *4.56 (1)* | *5.05 (1)* |
| *P-value* | *0.033* | *0.025* |
| *Intra-pony correlation (%)* | *12.4* | *12.8* |

**Table 12.10: Summary of multilevel linear regression modelling of airway inflammation (AI) score, including individual terms for *S. zooepidemicus* types**

| **Outcome variable** / Effect type / Explanatory variable in model | **Estimate (S.E.)** |
|---|---|
| **Airway inflammation score** | **Model 5 n=214** |
| Fixed effect | |
| Intercept | 3.63 (1.91) |
| AI 1 week previously | 0.30 (0.06) |
| AI 2 weeks previously | 0.21 (0.05) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.59 (0.17) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.09 (0.03) |
| $[Log_{10}$ cfu/ml A1 HVu$]^{-1}$ | 0.40 (0.14) |
| $[Log_{10}$ cfu/ml A1 HVu$]^{-0.5}$ | -4.26 (1.51) |
| $[Log_{10}$ cfu/ml C1 HV3$]^{-0.5}$ | 1.11 (0.59) |
| $Log_n[Log_{10}$ cfu/ml C1 HV3] | 1.83 (0.92) |
| $Log_{10}$ cfu/ml A1 HV4 | 0.16 (0.08) |
| Random effect | |
| Level 2 variance: Pony | 0.00 (0.00) |
| Level 1 variance: Observation | 1.92 (0.19) |
| *-2*loglikelihood* | *747.14* |
| *LRS $\chi^2$ (d.f.)* | *3.94 (1)* |
| *P-value* | *0.047* |
| *Intra-pony correlation (%)* | *0.0* |

Examination of pony-level residuals

Figures A3.4 and A3.5 represent caterpillar plots of the ranked pony-level residuals and their 95% confidence limits for the final multilevel regression models for clinical score including transferrin D and F2 haplotypes, respectively. Both figures illustrated that there were no significant departures of pony-level residuals as judged by 95% confidence intervals that did not include zero (hatched line) and were consistent with the non-significant pony-level random effect terms in both these final models (Table 12.9 and A3.2).

Figures A3.6 and A3.7 represent caterpillar plots of the ranked pony-level residuals and their 95% confidence limits for the final multilevel regression models for CDNS score including transferrin D and F2 haplotypes, respectively. The pony-level residuals for the model with transferrin haplotype D showed that pony 28 was the most extreme outlier only pony with a negative residual value i.e. had lower CDNS score than was predicted by the final model. For the final model including the transferrin F2 haplotype variable, pony 28

again had a significant negative residual and ponies 23 and 26 had significant positive residuals i.e. had higher CDNS scores than were predicted by the final model.

To assess the influence of the largest pony-level residuals on the fit of final models for CDNS score, models were refitted with data from the relevant ponies added to the fixed effect part of the models as a separate intercept and excluded from the pony-level random effects. Table A3.4 summarises the results of further modelling of CDNS score. Results are presented for the re-classification of pony 28 in both transferrin D (Model 5b) and F2 (Model 6b) models and for the reclassification of pony 23 (Model 7b) and pony 26 (Model 8b) in the transferrin F2 model.

Results of each stage in the further modelling of CDNS score showed that as the ponies with largest absolute value residuals were reassigned in the models there was a decrease in deviance and the overall fit of models improved significantly. In all except one modelling stage (reclassification of pony 26 in Model 8b) there was an increase in the absolute value (but in a negative direction for transferrin D) of the fixed effect estimates for the transferrin parameters. This was accompanied by a corresponding decrease in the standard errors for these estimates and hence the significance of their association with CDNS score also increased. In addition, there was a notable decrease in both the estimates and significance of pony level variances across the sequential modelling, which also resulted in a sequential decrease in intra-pony correlation. The estimates and standard errors for the autoregressive fixed effect parameters and observation level variances remained largely unaltered during this modelling process.

The final multilevel model (Model 5, Table 12.10) for airway inflammation was virtually identical to the final multiple linear regression model (Table 12.8). The model contained 2 autoregressive variables, a quadratic term for non-haemolytic *Streptococcus* spp. and various polynomial and linear terms for 3 *S. zooepidemicus* types in tracheal washes. The model had very small pony-level variance and consequently, did not have pony-

level residuals that significantly varied from zero. The model that included the autoregressive and non-haemolytic *Streptococcus* spp. quadratic term (Model 2) did have a small amount of non-significant pony-level variance although all pony-level residuals for this model had 95% confidence intervals that were small in value and included zero (Figure A3.8).

### 12.2.2 Individual clinical signs

#### *12.2.2.1 Univariable analyses*

Tables A3.5 - A3.9 summarise results of univariable ordinary logistic regression analyses of the association of different *S. zooepidemicus* types with the binary outcomes of nasal and ocular discharge, coughing, abnormal breathing/dyspnoea and SMLN enlargement respectively.

Results are presented for tracheal and nasopharyngeal isolates of each of the 5 most prevalent *S. zooepidemicus* types, with tracheal isolates represented separately as binary, ordered categorical and continuous outcomes and nasopharyngeal isolates as binary outcomes only.

Results in Table A3.5 indicated that only isolation of *S. zooepidemicus* type C1 HV3 in tracheal washes was associated with an increased risk of nasal discharge, with the significant ordered categorical and continuous results suggesting that risk increased with higher $\log_{10}$ cfu/ml values. Isolation of any of the 5 most prevalent *S. zooepidemicus* types from the nasopharynx was not shown to be significantly associated with increased risk of nasal discharge in these ponies.

Results in Table A3.6 indicated that isolation of any of the 5 most prevalent types from either the trachea or nasopharynx were not shown to be significantly associated with increased risk of ocular discharge in these ponies.

Results in Table A3.7 indicated that isolation of *S. zooepidemicus* type A1 HVu in tracheal washes and type A1 HV4 from the nasopharynx were associated with an increased risk of coughing. The significant results from ordered categorical and continuous outcomes suggested that risk of coughing increased with higher $\log_{10}$ cfu/ml values of A1 HVu in tracheal washes.

Results in Table A3.8 indicated that isolation of *S. zooepidemicus* types A1 HVu and A1 HV4 in tracheal washes and type A1 HV4 from the nasopharynx were associated with an increased risk of abnormal breathing/dyspnoea. The significant results from ordered categorical and continuous outcomes suggested that risk of abnormal breathing/dyspnoea increased with higher $\log_{10}$ cfu/ml values of both *S. zooepidemicus* types in tracheal washes.

Results in Table A3.9 indicated that isolation of *S. zooepidemicus* type C1 HV3 from the trachea and nasopharynx were both associated with an increased risk of SMLN enlargement. The significant results from ordered categorical and continuous outcomes suggested that risk of SMLN enlargement increased with higher $\log_{10}$ cfu/ml values of this *S. zooepidemicus* type in tracheal washes.

#### *12.2.2.2 Multivariable analyses*

Table 12.11 summarises the final multivariable logistic regression models that include pony-level random effects and for illustration of principal purposes the most significant terms for individual *S. zooepidemicus* types for each of the individual clinical outcomes of nasal discharge, coughing, abnormal breathing/dyspnoea and SMLN enlargement. Results of multivariable logistic regression analyses incorporating pony-level random effects are now discussed for each of the individual clinical signs.

**Table 12.11: Summary of final multivariable logistic regression models including pony-level random effects and most significant terms for individual *S. zooepidemicus* types for individual clinical outcomes**

| Clinical outcome being modelled (n) | Egret software maximum likelihood estimates (MLE) | | | | |
|---|---|---|---|---|---|
| Explanatory variables in model | β | S.E. β | Odds ratio | 95% CI | P-value |
| **Nasal discharge (n=194)** | | | | | |
| Intercept | -0.95 | 0.53 | | | |
| Nasal discharge 1 week previously | 0.41 | 0.48 | 1.51 | 0.59 – 3.89 | 0.394 |
| Nasal discharge 2 weeks previously | 0.14 | 0.45 | 1.16 | 0.48 – 2.79 | 0.748 |
| Nasal discharge 3 weeks previously | 1.19 | 0.37 | 3.29 | 1.60 – 6.73 | 0.001 |
| NP *B. bronchiseptica* | 0.86 | 0.43 | 2.37 | 1.03 – 5.47 | 0.043 |
| TW C1 HV3 *S. zooepidemicus* isolated | 1.04 | 0.75 | 2.83 | 0.65 – 12.4 | 0.165 |
| Pony level random effects term | 0.57 | 0.53 | | | |
| **Coughing (n=238)** | | | | | |
| Intercept | -5.06 | 0.96 | | | |
| Coughing 1 week previously | 1.34 | 0.54 | 3.81 | 1.33 – 10.9 | 0.013 |
| Vaccine group: placebo | 2.40 | 0.79 | 11.0 | 2.31 – 52.0 | 0.003 |
| Vaccine group: late introduction | -0.03 | 0.92 | 0.97 | 0.16 – 5.91 | 0.976 |
| $<10^3$ cfu/ml TW A1 HV3 *S. zooepidemicus* | 0.64 | 1.31 | 1.90 | 0.14 – 24.9 | 0.625 |
| $>10^3$ cfu/ml TW A1 HV3 *S. zooepidemicus* | 1.69 | 0.78 | 5.41 | 1.17 – 25.0 | 0.030 |
| $<10^3$ cfu/ml TW A1 HV u *S. zooepidemicus* | 0.30 | 0.94 | 1.35 | 0.21 – 8.56 | 0.750 |
| $>10^3$ cfu/ml TW A1 HV u *S. zooepidemicus* | 2.96 | 0.78 | 19.2 | 4.13 – 89.3 | <0.001 |
| NP A1 HV4 *S. zooepidemicus* isolated | 1.53 | 0.69 | 4.60 | 1.20 – 17.7 | 0.026 |
| Transferrin F2 | 1.77 | 0.72 | 5.86 | 1.44 – 23.8 | 0.013 |
| Pony level random effects term | 0.86 | 0.38 | | | |
| **Abnormal breathing/dyspnoea (n=218)** | | | | | |
| *Model 1* | | | | | |
| Intercept | -0.32 | 0.54 | | | |
| Dyspnoea 1 week previously | 0.30 | 0.47 | 1.35 | 0.54 – 3.39 | 0.520 |
| Dyspnoea 2 weeks previously | 1.07 | 0.42 | 2.92 | 1.27 – 6.71 | 0.012 |
| $<10^3$ cfu/ml TW C1 HV3 *S. zooepidemicus* | 2.99 | 1.44 | 20.0 | 1.19 – 336 | 0.038 |
| $>10^3$ cfu/ml TW C1 HV3 *S. zooepidemicus* | 0.35 | 0.77 | 1.41 | 0.32 – 6.34 | 0.650 |
| $<10^3$ cfu/ml TW A1 HV4 *S. zooepidemicus* | -0.53 | 1.93 | 0.59 | 0.01 – 26.1 | 0.785 |
| $>10^3$ cfu/ml TW A1 HV4 *S. zooepidemicus* | 1.39 | 0.81 | 4.02 | 0.82 – 19.7 | 0.087 |
| Transferrin D | -1.66 | 0.62 | 0.19 | 0.06 – 0.64 | 0.007 |
| Pony level random effects term | 0.96 | 0.41 | | | |
| *Model 2* | | | | | |
| Intercept | -2.21 | 0.42 | | | |
| Dyspnoea 1 week previously | 0.19 | 0.46 | 1.21 | 0.49 – 2.96 | 0.677 |
| Dyspnoea 2 weeks previously | 0.98 | 0.41 | 2.68 | 1.19 – 6.03 | 0.018 |
| $<10^3$ cfu/ml TW C1 HV3 *S. zooepidemicus* | 2.81 | 1.49 | 16.7 | 0.90 – 309 | 0.059 |
| $>10^3$ cfu/ml TW C1 HV3 *S. zooepidemicus* | 0.07 | 0.76 | 1.07 | 0.24 – 4.75 | 0.928 |
| $<10^3$ cfu/ml TW A1 HV4 *S. zooepidemicus* | -0.33 | 1.94 | 0.72 | 0.02 – 32.1 | 0.864 |
| $>10^3$ cfu/ml TW A1 HV4 *S. zooepidemicus* | 1.34 | 0.81 | 3.82 | 0.78 – 18.6 | 0.097 |
| Transferrin F2 | 1.93 | 0.63 | 6.90 | 2.02 – 23.6 | 0.002 |
| Pony level random effects term | 0.92 | 0.38 | | | |
| **SMLN enlargement (n=244)** | | | | | |
| Intercept | -1.87 | 0.42 | | | |
| SMLN enlargement 1 week previously | 1.60 | 0.45 | 4.97 | 2.07 – 11.9 | <0.001 |
| Vaccine group: placebo | 0.26 | 0.55 | 1.30 | 0.44 – 3.78 | 0.636 |
| Vaccine group: late introduction | 1.77 | 0.76 | 5.85 | 1.33 – 25.8 | 0.019 |
| $<10^3$ cfu/ml TW C1 HV3 *S. zooepidemicus* | -0.06 | 1.38 | 0.94 | 0.06 – 14.1 | 0.964 |
| $>10^3$ cfu/ml TW C1 HV3 *S. zooepidemicus* | 1.26 | 0.78 | 3.51 | 0.76 – 16.2 | 0.107 |
| Pony level random effects term | 0.90 | 0.38 | | | |

Nasal discharge

The final logistic regression model presented for nasal discharge included all 3 autoregressive variables, binary terms for type C1 HV3 type *S. zooepidemicus* isolated in tracheal washes (not statsitically significant) and *Bordetella bronchiseptica* recovered from the nasopharynx and a pony-level random effect term.

Examination of the final model showed that among the autoregressive variables, a nasal discharge 3 weeks previously was significantly associated with a greater than 3-fold increased risk of the same sign in any given week. The autoregressive variables for nasal discharge one and 2 weeks previously were retained *a priori*, irrespective of whether they themselves were significantly associated with the clinical outcome. Controlling for the other variables in the final model, isolation of *Bordetella bronchiseptica* from the nasopharynx was found to be associated with an increased risk (adjusted OR 2.4, P=0.043) of nasal discharge. Although not demonstrated to be statistically significant at the 5% level the best evidence for an association between an individual *S. zooepidemicus* type and nasal discharge was for isolation of type C1 HV3 from tracheal washes, for which there was an increased but non-significant risk (OR 2.8, P=0.165).

As these data were highly correlated within individual ponies all final models were produced *a priori* including a pony-level random effects term in order to account for random variability between ponies. However, in this final model of nasal discharge including all 3 autoregressive variables there was no evidence for significant random variability between ponies.

Coughing

Logistic regression analysis of the binary clinical outcome of coughing resulted in a final model which included the autoregressive variable of coughing the previous week, vaccine group category, categorical classifications of *S. zooepidemicus* types A1 HV3 and

A1 HVu in tracheal washes, a binary variable for isolation of A1 HV4 from the nasopharynx and a pony-level random effect term.

With inclusion of a significant pony-level random effect term there was evidence of an increased risk of coughing in any given week when ponies had coughed the previous week, was administered placebo rather than vaccine, had $>10^3$ cfu/ml of either type A1 HV3 or particularly type A1 HVu *S. zooepidemicus* isolated from tracheal washes, had type A1 HV4 *S. zooepidemicus* isolated from the nasopharynx or possessed transferrin haplotype F2.

Abnormal breathing/dyspnoea

As with the results of multivariable analyses of aggregated clinical outcome scores, 2 final models, corresponding to inclusion of either transferrin D (Model 1) or F2 (Model 2) haplotypes, were derived for the logistic regression analysis of the binary clinical outcome of abnormal breathing/dyspnoea. Other significant or marginally significant and/or *a priori* variables in the final models included the autoregressive variable of abnormal breathing/dyspnoea one (*a priori)* and 2 weeks (significant) previously, categorical classifications of *S. zooepidemicus* types C1 HV3 and A1 HV4 in tracheal washes (marginal) and pony-level random effect term (*a priori* and significant).

With inclusion of a significant pony-level random effect term there was evidence of an increased risk of abnormal breathing/dyspnoea in any given week when ponies had abnormal breathing/dyspnoea 2 weeks previously, had $<10^3$ cfu/ml of type C1 HV3 or $>10^3$ cfu/ml of type A1 HV4 *S. zooepidemicus* isolated from tracheal washes or possessed transferrin haplotype F2. There was evidence from model 1 that ponies that possessed transferrin haplotype D were at decreased risk of abnormal breathing/dyspnoea.

SMLN enlargement

The final logistic regression model for SMLN enlargement included the autoregressive variable for clinical outcome the previous week, vaccine group category, a categorical classification of *S. zooepidemicus* types C1 HV3 in tracheal washes and a pony-level random effect term.

With inclusion of a significant pony-level random effect term there was evidence of an increased risk of SMLN enlargement among ponies that were affected the previous week (adjusted OR 5.0, P<0.001) and in ponies that were introduced later compared to vaccinated animals (adjusted OR 5.9, P=0.019). Although not demonstrated to be statistically significant at the 5% level the best evidence for an association between an individual *S. zooepidemicus* type and SMLN enlargement was for isolation of $>10^3$ cfu/ml type C1 HV3 from tracheal washes, for which there was an increased but non-significant risk (OR 3.5, P=0.107).

# CHAPTER 13

# DISCUSSION

## 13.1 *S. zooepidemicus* types identified in this study

Results from this study indicated that compared to the number of ponies (n=29) there were a relatively large number of different types of *S. zooepidemicus* (n=39) isolated during the few weeks of intensive sampling. However, there was a marked variation in the prevalence of these different types, with a relatively small number of types (n=10) accounting for a large proportion of all isolates (84%) and many types being isolated relatively infrequently.

The findings of highly variable prevalence of different types was consistent with different *S. zooepidemicus* types having variable dominance during the period of sampling. That is the most prevalent types appeared to become established as dominant types and were isolated much more frequently than some other types, which did not appear to become established. In this context it is important to note that typing of multiple colonies of *S. zooepidemicus* from the same samples in a parallel study in Thoroughbreds demonstrated that they were of the same type (R. Laxton & N. Chanter, unpublished observations). This was consistent with the findings of Timoney *et al.* (1997) and confirmed that *S. zooepidemicus* isolates from respiratory tract infections are usually single clones.

There was also generally similar overall prevalence of different types isolated from both nasopharyngeal and tracheal samples (Figure 12.1) and a very similar pattern of prevalence of isolates of the same type from these 2 different sites in the respiratory tract over the course of the study (Figure 12.2). However, different *S. zooepidemicus* types, whether from the trachea or nasopharynx, demonstrated markedly different patterns of prevalence over the course of the study, with some types (e.g. A1 HV1 and C1 HV3)

demonstrating declining prevalence and others (e.g. A1 HVu and A1 HV2/3) showing increasing prevalence during the 10 weeks of consecutive sampling.

Taken together these results provide strong evidence for clonal succession of *S. zooepidemicus* types among groups of ponies. The evidence for such a phenomenon in individual animals is at this stage (data not shown), however, less convincing due to the large number of individual types isolated from most animals during the course of the study relative to the limited number of sampling occasions for each animal.

This study identified novel *S. zooepidemicus* types that had not been apparently recognised by the original authors of the 2 typing techniques that were simultaneously adopted in this study, although a much larger number of isolates were typed in the current study. PCR of the M-protein hypervariable region demonstrated that 16% and 18.6% of tracheal and nasopharyngeal isolates, respectively, were of a type or types other than the 5 regions described by Walker & Timoney (1998). Isolates with these untyped hypervariable regions have been shown to possess the M-protein gene and produce a product of appropriate size when PCR using SzPN and SzPNC3 primers is applied to them (R. Laxton & N. Chanter, unpublished observations). Work remains to be done to sequence these untyped hypervariable regions and to demonstrate whether or not they are all of the same novel type or comprise several different types, which would potentially identify many more specific *S. zooepidemicus* types among the isolates from this and other similar studies.

This study also demonstrated that some isolates of *S. zooepidemicus* possessed polymorphism of either the M-protein hypervariable region or 16S-23S RNA gene intergenic spacer and this also contributed greatly to the increased number of different *S. zooepidemicus* types identified during the study. Results also showed that a far larger proportion of isolates had polymorphism of the HV region (23.8%) than the intergenic spacer (6.3%) and this was actually a larger proportion of isolates than any other single HV region type. Subsequent PCR and Southern blotting investigations using isolates from triple

repeated single colony subcultures confirmed the presence of multiple copies of different M-protein HV region genes and 16S-23S RNA intergenic spacers (R.Laxton & N. Chanter, unpublished observations).

This study demonstrated that slightly more *S. zooepidemicus* types were isolated from the trachea (n=33) than the nasopharynx (n=27) and a similar finding was observed in a parallel study in young Thoroughbreds in training (22 types isolated from tracheal washes and 14 types from nasopharyngeal swabs). In addition, there was a larger proportion of positive isolations of *S. zooepidemicus* from the trachea (94%) than the nasopharynx (87%). These findings appear somewhat counter to the observation by Timoney *et al,* (1997) that multiple clones of *S. zooepidemicus* were demonstrated in the tonsils of the upper respiratory tract and single clones, all of which were represented among the tonsillar isolates, were isolated from lungs of pneumonia cases. From their observations, Timoney *et al.* (1997) hypothesised that lower airway infection with *S. zooepidemicus* was an endogenous opportunistic infection (presumably from a tonsillar source) by a single clone. Although tonsils were not specifically sampled in the current study, it may have been expected that there would be a gradient of infection to the lower airways, with swabs from the nasopharynx detecting more types due to the proximity of the tonsils to the nasopharyngeal sampling site and less types identified in the trachea and lungs. If it is a true finding that more types are isolated from the lower airway than the upper, this might be suggestive of 2 issues. Firstly, there are possible differences in the conditions for colonisation between the upper and lower airways and this is possibly related to anatomical and physiological differences between these sites. For instance, the difference in proximity and extent of lymphoid tissue in these different sites may be important and permits the preferential persistence of more types more frequently in the lower respiratory tract than the upper respiratory tract. Secondly, the ability of the lower and upper respiratory tracts to maintain *S. zooepidemicus* of different types to different extents might be consistent with

the source of such infections actually being extraneous (i.e. from the environment) and not necessarily endogenous in all cases.

Examination of data for *S. zooepidemicus* isolates with specific intergenic spacer types but ignoring their HV region types, showed that the most prevalent type was A1, which comprised two-thirds of all isolates. The second most prevalent intergenic spacer type was C1, which comprised only 18% of all isolates and in this series of samples representatives of some intergenic spacer types were not isolated, including B2, C2 and D2 types. Among specific HV region types ignoring their intergenic spacer types, there was a greater equality of prevalence among the 2 most prevalent (HV1 22.5% and HV3 21.3%) and the 2 least prevalent (HV2 5.9% and HV4 6.7%) individual types. A large proportion of all isolates (41%) were either polymorphic or untyped (HVu) by the PCRs used in this study and no HV5 region types were isolated at all in this study.

With hindsight, it would have been beneficial if all the ponies had been sampled before the group of 5 'late introductions' had been mixed with the 24 other ponies from a geographically restricted area and moved to Newmarket. This would have established the probable origin of sutypes of *S. zoopeidemicus* that were later identified. This would have made it clearer as to which types probably came from the 'late introductions' and which were met in the environment at the new premises in Newmarket.

## 13.2 Vaccine strains of *S. zooepidemicus*

The strains of *S. zooepidemicus* included in the vaccine were not typed by the PCR methods used in this study prior to their inclusion in the vaccine. Strains of *S. zooepidemicus* were selected by using the opsonins naturally occurring in AHT ponies, essentially by the methods of Causey *et al.* (1995), further supplemented with studies of cross-opsonisation using rabbit antisera raised to hot acid extracts of a series from temporally and geographically disparate sources. The 2 isolates selected for the vaccine

were those exhibiting the least cross-opsonisation. Subsequent typing identified the vaccine strains as D2 HV3 and A1 HV2/5, which with completion of all typing of *S. zoopidemicus* isolates were found to have an overall prevalence among 538 typed isolates of 0% and <0.2%, respectively. This indicated that by M-protein HV region and 16S-23S intergenic spacer PCR typing techniques, the 2 specific vaccine strains were not represented (except for a single isolate from a vaccinated pony) even among non-vaccinated animals in this study. However, one vaccine *S. zooepidemicus* type was of the most frequently isolated intergenic spacer type (A1 HV2/5) and the other was of the second most prevalent specific single HV region type (D2 HV3). This indicates that typing of an increased number of representative equine isolates from different, discrete populations of horses is required so that a consensus as to the most appropriate individual types can be achieved. This would then allow inclusion in future vaccines of the important virulence determinants in these prevalent types, rather than unrepresentative types.

The degree of immunological cross-protection between different subtypes of *S. zooepidemicus* is not known and therefore it is difficult to properly assess the ability of the vaccine in the study to protect against infection with different subtypes of the same bacteria. However, it is probable from the wide range of subtypes isolated from ponies with overt clinical signs of respiratory disease that there was little cross-protection from vaccination, although the apparent failure of the vaccine may have occurred for several reasons. Ponies may have had previous exposure to the vaccine subtypes and were already protected against them or there may have been no natural exposure to the vaccine subtypes during the study and as such the vaccine was not actually challenged.

## 13.3 Association of *S. zooepidemicus* types with disease

There is some evidence that in this population of ponies during the period of study that not all of the 5 most prevalent types of *S. zooepidemicus* demonstrated an equivalent

degree of association with disease. This was illustrated in the variation in slopes of the regression lines from the best fitting, 2-power polynomial regression models of outcome scores plotted against $\log_{10}$ cfu/ml of specific *S. zooepidemicus* types. This was particularly evident for the associations of types A1 HV1 and A1 HV4 with clinical scores (Figure A3.1). This impression was confirmed with the inclusion of different *S. zooepidemicus* types in multivariable regression and multilevel modelling. In each of the final models using these different analysis techniques, *S. zooepidemicus* type A1 HV1 was not retained as being statistically significantly associated with any of the 3 outcomes of interest after controlling for other factors including autoregressive variables, transferrin type and other *S. zooepidemicus* types.

With individual clinical signs multivariable analyses suggested that after controlling for other factors there were some differences in the association of different *S. zooepidemicus* types with specific clinical signs. There was evidence that types A1 HV3 and A1 HVu were more strongly associated with coughing whereas types C1 HV3 and A1 HV4 were associated with abnormal breathing/dyspnoea. Care is required though in the interpretation of many of these final models as several variables for infection by different *S. zooepidemicus* subtypes were not statistically significantly associated with outcomes at the 5% level but were retained for illustration of principal purposes only.

However, considerable care needs to be taken in interpreting these apparent differences in association between different *S. zooepidemicus* types and disease measures as they may not necessarily reflect true differences in the virulence of different types. This is because there are differences in the previous infectious burdens and consequential immune responses of each pony, reflecting likely differences in previous exposure to different types. In addition, there would have been acquisition of immunity to different types during the course of the study period, albeit that this period was only a few weeks. Therefore, it might be expected that the more prevalent types in the earlier weeks of sampling (e.g. type A1

HV1) would result in being the most prevalent types overall. With ponies developing immunity over the period, this would also result in them having apparently reduced virulence compared with isolates that increased in prevalence at a later stage in the study period and for which less immunity would have developed. This is again strong evidence for clonal succession.

However, given the ubiquitous nature of *S. zooepidemicus* infection in horses, experimental infections in specific-pathogen-free (SPF) foals would be required to definitively demonstrate that there were true differences in the virulence of different *S. zooepidemicus* types (type-specific virulence). From what is known already about likely determinants of virulence in *S. zooepidemicus* and *S. equi*, it is probable that the HV region of the M-protein would be more predictive of virulence than would the 16S-23S RNA gene intergenic spacer, although there will be undoubtedly other important determinants that vary between isolates possessing the same HV region and which may possibly be linked to intergenic spacer type. The current identification of many different potential virulence determinants arising from the sequencing of the genome of *S. equi* (Carl Robinson, personal communication) and cross-checking for their presence or absence in isolates of *S. zooepidemicus*, may allow some future prediction of likely virulence in the absence of challenge infections in SPF foals. In addition, consistency between *S. zooepidemicus* types associated with disease in different animal populations may also help to identify more virulent types. In this regard results of similar typing techniques applied in Thoroughbred racehorses (unpublished observation) and even kennels of dogs suffering severe respiratory disease associated with *S. zooepidemicus* infection (V. Chalker, RVC, personal communication) may confirm some of the findings of this study.

# SECTION 5

# CONCLUSIONS AND FUTURE WORK

# CHAPTER 14

# CONCLUSIONS AND FUTURE WORK

## 14.1 Conclusions

### 14.1.1 Clinical respiratory disease in Thoroughbred racehorses

A matched case control study demonstrated that clinically apparent respiratory disease in young Thoroughbred racehorses was statistically associated with several infectious and non-infectious risk factors. Different comparisons were made between cases and controls with and without subclinical disease and controls with sub-clinical disease were compared to those with none:

i) Horses that had *Actinobacillus/Pasteurella* spp. or *Mycoplasma felis* isolated from the trachea were at increased risk of suffering clinical respiratory disease.

ii) *Streptococcus zooepidemicus* in the trachea was significantly associated with both subclinical inflammatory airway disease (IAD) in controls and clinical respiratory disease in cases when compared with controls with no endoscopic or cytological evidence of IAD.

iii) The only significant upper respiratory tract bacterial infection associated with clinical respiratory disease was *Actinobacillus/Pasteurella* spp., which was identified only when clinical cases were compared with horses with no endoscopic or cytological evidence of IAD.

iv) There was no significant association found between viral infections and clinical disease other than for equine influenza, which was an extremely rare infection in this well vaccinated population.

v) Younger horses were at increased risk of suffering clinical respiratory disease, particularly yearlings.

vi) Horses that had entered training within the last 3 months were at increased risk of suffering clinical respiratory disease.

vii) Although there was clear potential for confounding between age and recent entry into training (e.g. yearlings have invariably entered training within the last 3 months), simultaneous inclusion of these variables suggested significant independent effects.

In conclusion, this case control study demonstrated that risk of clinical respiratory disease in flat trained racehorses in the United Kingdom decreased as horses grew older, and spent more time in training. These findings suggest that horses somehow develop resistance to the disease following exposure. A limited number of bacterial and mycoplasma infections of the trachea and equine influenza virus infection were important risk factors for clinical presentation of respiratory disease and development of resistance to these infections might explain the reduction in disease in older, longer trained animals.

### 14.1.2 Respiratory disease in Welsh Mountain ponies

The efficacy of an experimental bacterial vaccine was evaluated for its effect on natural respiratory disease in recently weaned and transported Welsh Mountain pony foals using a blinded and randomised, controlled trial.

Repeated weekly clinical evaluations and microbiological samplings were conducted in 12 ponies that received vaccine, 12 ponies that received placebo and 5 other ponies that were added after vaccination with the intention of introducing additional infections into the group. Both pony-level and observation-level analyses were conducted on study data.

Isolates of *S. zooepidemicus* from the trachea and nasopharynx were subjected to molecular typing by 2 PCR assays. The hypervariable (HV) region of the M-protein (5 possible types) and the 16S-23S RNA gene intergenic spacer (8 possible types) were typed.

i) Pony-level and observation-level analyses using aggregated clinical scores as the dependent variable did not demonstrate any significant effect from vaccination on respiratory disease. However, among individual signs there was evidence for significantly reduced coughing among vaccinated ponies compared with those that received placebo. The vaccine was not associated with a reduction in the burden of infection with *S.zooepidemicus* or Actinobacillus/*Pasteurella* spp. The apparent failure of the vaccine to protect against infection with *S. zooepidemicus* may have occurred for several reasons. The study ponies may have had previous exposure to the vaccine subtypes and were already protected against them. Alternatively there may have been no natural exposure to the vaccine subtypes during the study and as such the vaccine was not actually challenged. If this was the case, however, it would suggest that there was little cross-protection conveyed between the vaccine and natural challenge subtypes.

ii) Infection of the trachea with *S. zooepidemicus* was an important risk factor for aggregated clinical scores and some individual clinical signs (nasal discharge, cough and abnormal breathing/dyspnoea). There was evidence for a significant dose response between *S. zooepidemicus* infection and dyspnoea and airway inflammation. There was only limited evidence for high numbers of *Pasteurella* spp. in tracheal washes being associated with clinical signs. These findings were largely consistent with those of previous studies of respiratory disease in young racehorses, although the limited sampling frame and relative scarcity of *Pasteurella* spp. may have contributed to some apparent inconsistency with these studies.

iii) Observation-level data were analysed using both autoregressive outcomes and use of pony-level random effects. There were significant pony-level random effects for aggregated clinical scores but not for airway inflammation score and there was

variation in the significance of pony-level random effects for different individual clinical signs. Models generally included significant autoregressive variables.

iv) Pony-level analyses demonstrated significant variation between cumulative aggregated clinical scores and *S. zooepidemicus* infectious measures (i.e. summary measures of aggregated clinical scores and *S. zooepidemicus* infections) between ponies with different transferrin haplotypes. Compared to ponies that did not possess these haplotypes, ponies with haplotype D had significantly lower clinical and infectious scores and those with haplotype F2 had significantly higher clinical and infectious scores. Observation-level analyses also demonstrated significant associations between aggregated clinical scores, coughing and abnormal breathing/dyspnoea and transferrin haplotypes D and F2. Haplotypes D and F2 had opposing directions of effect, which were consistent with those seen in pony-level analyses.

v) Examination of data from the molecular typing of respiratory isolates of *S. zooepidemicus* from ponies in this study may indicate clonal succession of types over time. There is a need in future to look at time series of infections in individual animals (e.g. using trellis plots) in order to try and properly assess clonal succession of infections by different subtypes of *S. zooepidemicus.*

vi) Novel *S. zooepidemicus* types were identified during the typing of isolates for this study (R. Laxton & N. Chanter, unpublished observations). These included previously untyped hypervariable region(s) (HVu) and intra-strain polymorphisms of both the HV region and intergenic spacer types. These findings indicate that the total possible number of *S. zooepidemicus* types from the simultaneous application of these 2 typing techniques could theoretically exceed the originally envisaged total of 40.

vii) There were more *S. zooepidemicus* types isolated from the trachea than the nasopharynx.

viii) Although there were apparent differences in the strength of association of different *S. zooepidemicus* types with respiratory disease, considerable care is required in interpreting these findings. Further studies are required before drawing any firm conclusions (see below).

In conclusion, this study demonstrated that clinical respiratory disease in Welsh Mountain pony foals was associated with respiratory *S. zooepidemicus* infection, irrespective of any effect from the experimental vaccine being evaluated. Significant differences in clinical scores and *S. zooepidemicus* infections between ponies was associated with possession of particular transferrin haplotypes. Ponies with transferrin haplotypes D were at signifiantly decreased risk of disease and infection, whereas those with haplotype F2 were at significantly increased risk.

## 14.2 Future work

This thesis has improved our knowledge of infectious and non-infectious factors associated with equine respiratory disease. However, a more complete understanding of the disease and its aetiology is needed. In particular, further investigations should include the following:

i) Development of PCR typing techniques for accurately identifying *Actinobacillus/Pasteurella* spp. and their application in future epidemiological studies. Ward *et al.* (1998) demonstrated the inaccuracy of existing typing methods and consequently studies of the association with respiratory disease and these bacterial species have remained non-specific. This may lead to a significant

underestimation of the strength of association of some specific bacterial species. This also leads onto studies of type specific immunity.

ii) Prospective and specific investigation of the effects of travel and racing on clinical and subclinical respiratory disease in racehorses in the United Kingdom may be warranted. Possible covariates worthy of examination would include the distance and time spent travelling, posture during travel and various racing variables particularly including surface and going types.

iii) The significant differences in measures of respiratory disease between ponies possessing different transferrin haplotypes identified in this study appear novel and as such require confirmation with respect to their repeatability in other groups of horses. To this end routine prospective data collection has been initiated at the AHT in order to systematically monitor clinical respiratory disease in Welsh Mountain pony foals within a few months of their arrival from Wales. These data will be analysed for evidence of significant differences in clinical respiratory disease between different transferrin haplotypes and with sufficient numbers of animals it is hoped to better elucidate differences between ponies of specific transferrin phenotypes. In addition, these investigations could easily be extended to examine retrospective or prospective data on respiratory disease in Thoroughbred racehorses in training yards that keep good clinical records, as transferrin haplotypes are readily available through routine blood typing. Retrospective analyses have been performed with data from a longitudinal study of respiratory disease in racehorses and results are consistent with those found in the pony study in this thesis.

iv) Investigation of whether the apparently significant effect of transferrin haplotype is due to a direct mechanism of action or is due to an alternative mechanism manifested through a gene closely linked to the equine transferrin locus, is required. Examination of genes in the region of the transferrin locus in other mammalian

species with typed genomes (e.g. man, mouse, dog) may reveal likely candidates that offer an alternative explanation for these data. These candidates could then be specifically identified in the equine genome for their proximity to the transferrin locus and their association with disease specifically examined.

v) Studies to characterise iron binding in the streptococci of clinical importance in horses (*S. equi*, *S. zooepidemicus* and equine isolates of *S. pneumoniae* capsule type 3) are warranted.

vi) Possible differences in the conditions of colonisation by *S. zooepidemicus* between the upper and lower respiratory tract should be investigated.

vii) Characterisation of *S. zooepidemicus* types involved with respiratory disease should be extended to other populations such as Thoroughbred racehorses. To this end *S. zooepidemicus* isolates from a small prospective study in 3 Newmarket training yards have been typed already and are awaiting analysis of their association with inflammatory airway disease.

viii) The association of specific *S. zooepidemicus* types with respiratory disease requires further investigation. Ideally this should be done through type-specific *S. zooepidemicus* challenge infections in SPF foals, although such animals are not currently available. In the absence of SPF foals, characterising likely virulence determinants in *S. zooepidemicus*, identified through the *S. equi* genome project, may provide a preliminary means of predicting virulence.

In conclusion, this is a novel and exciting area of research for the horse, which in future collaborations with specialists in microbial pathogenesis including iron acquisition, equine and microbial genetics and infectious disease molecular epidemiology, should bring closer the prospect of practical methods for preventing bacterial respiratory disease in horses.

# SECTION 6

# REFERENCES & APPENDICES

# REFERENCES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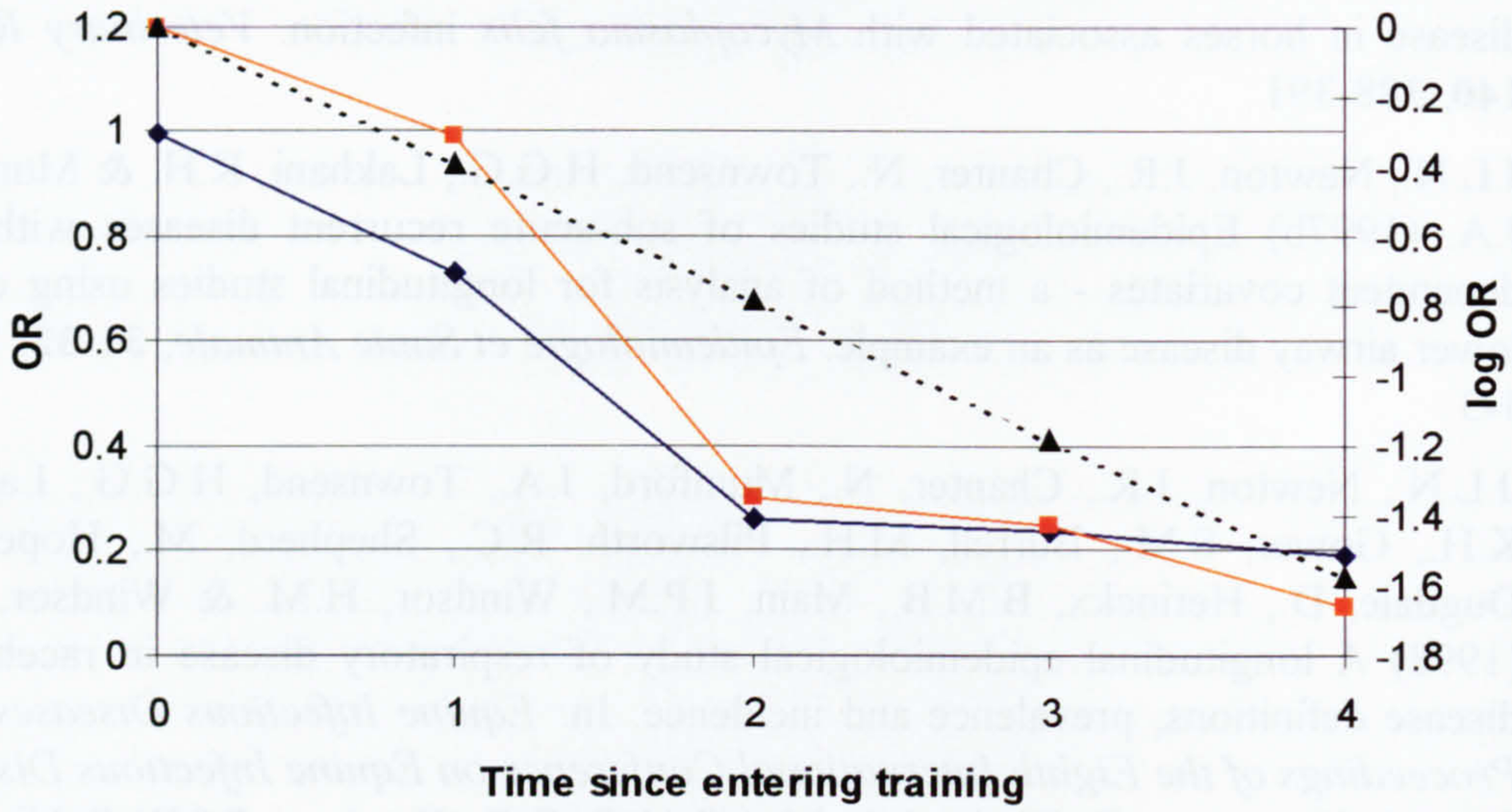
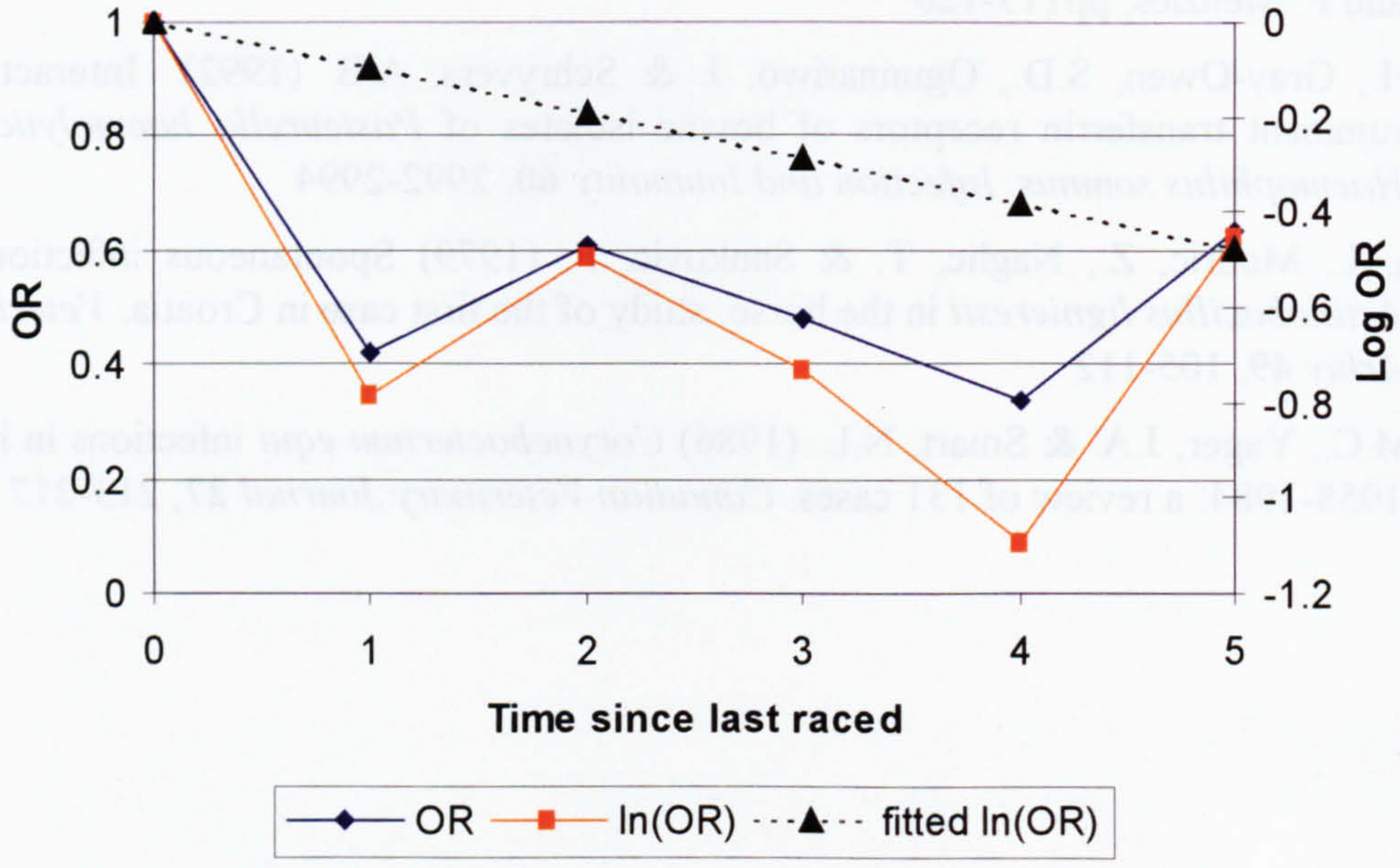
# APPENDIX 1

## Relationship between clinical respiratory disease in racehorses and time in training and time since last racing

### Figure A1.1: Relationship between time in training and odds of clinical disease




### Figure A1.2: Relationship between time since last raced and odds of clinical disease



# Relationship between tracheal bacteria and mycoplasma and clinical respiratory disease in racehorses

**Figure A1.3: Relationship between *S. zooepidemicus* infection and odds of clinical disease**

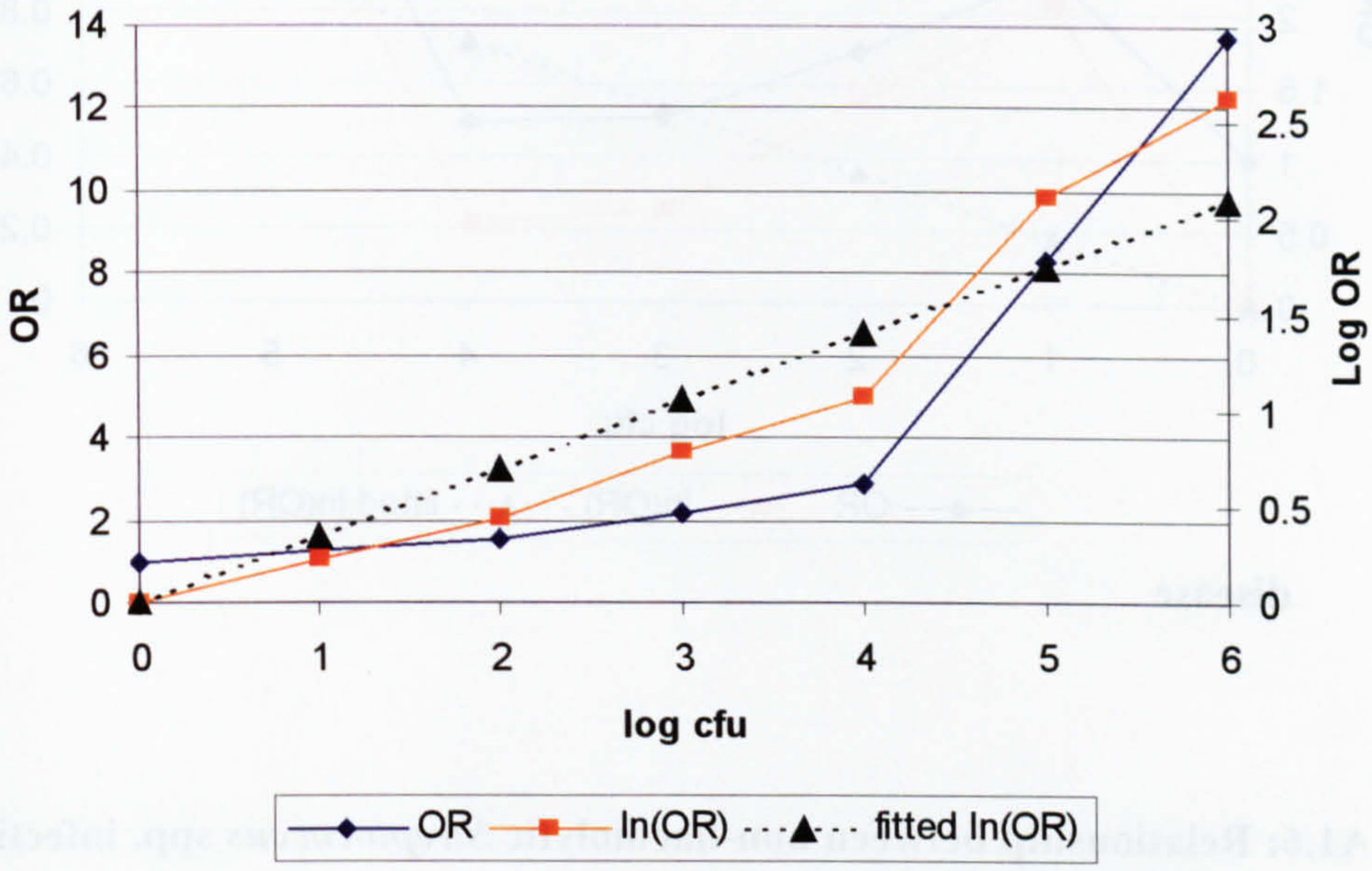



**Figure A1.4: Relationship between *Actinobacillus/Pasteurella* spp. infection and odds of clinical disease**

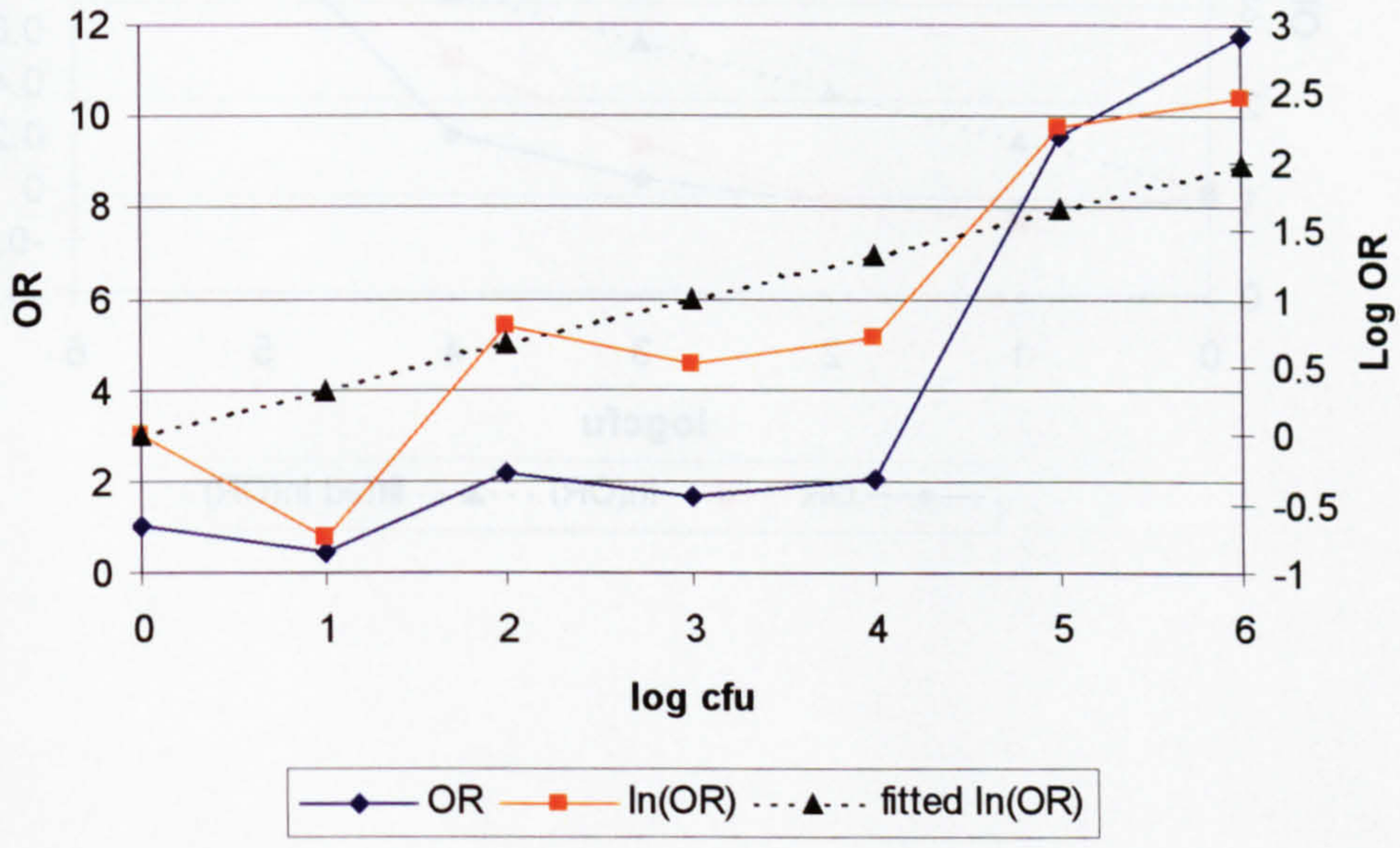


**Figure A1.5: Relationship between *S. pneumoniae* infection and odds of clinical**

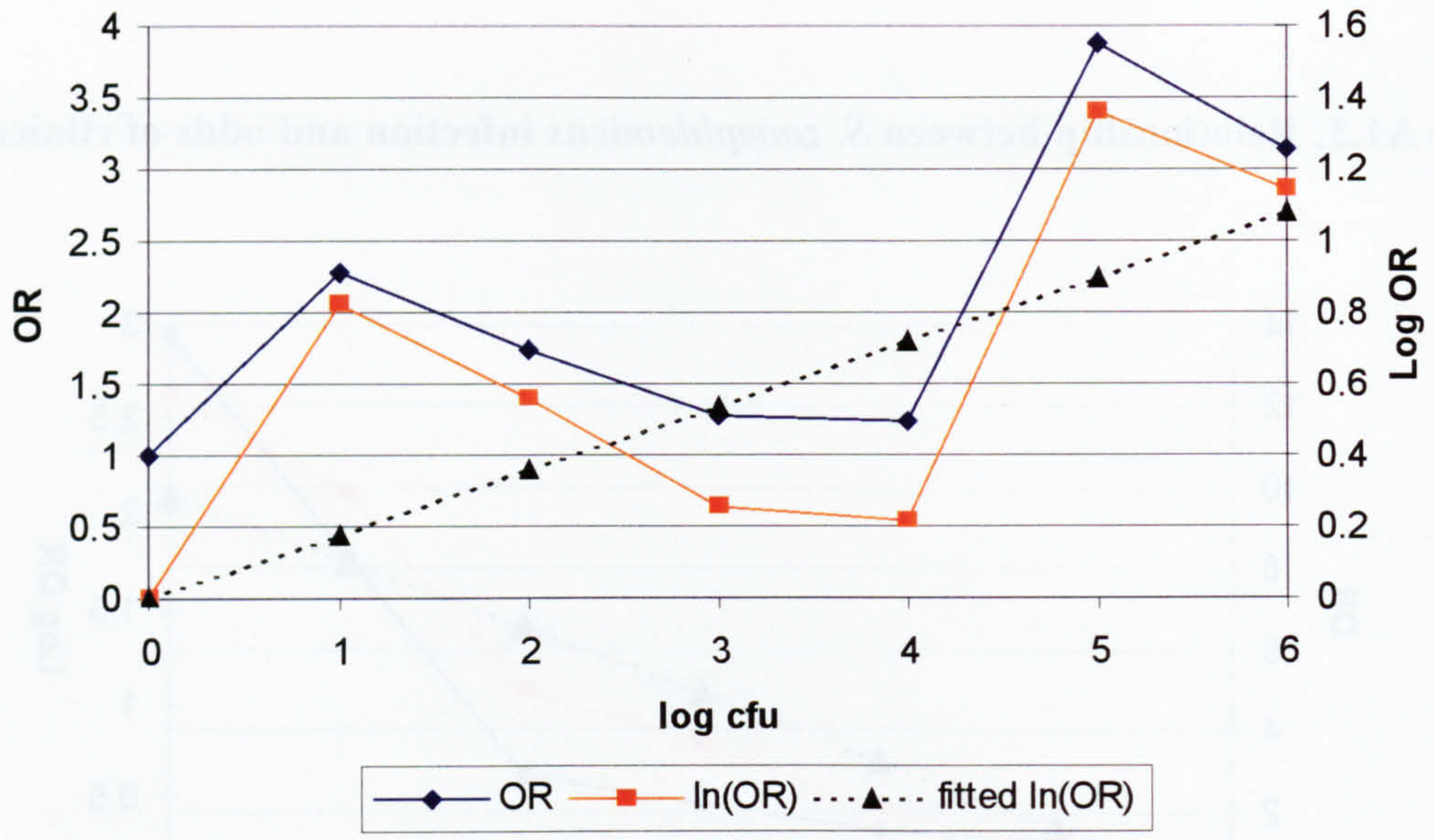



**Figure A1.6: Relationship between non-haemolytic *Streptococcus* spp. infection and odds of clinical disease**

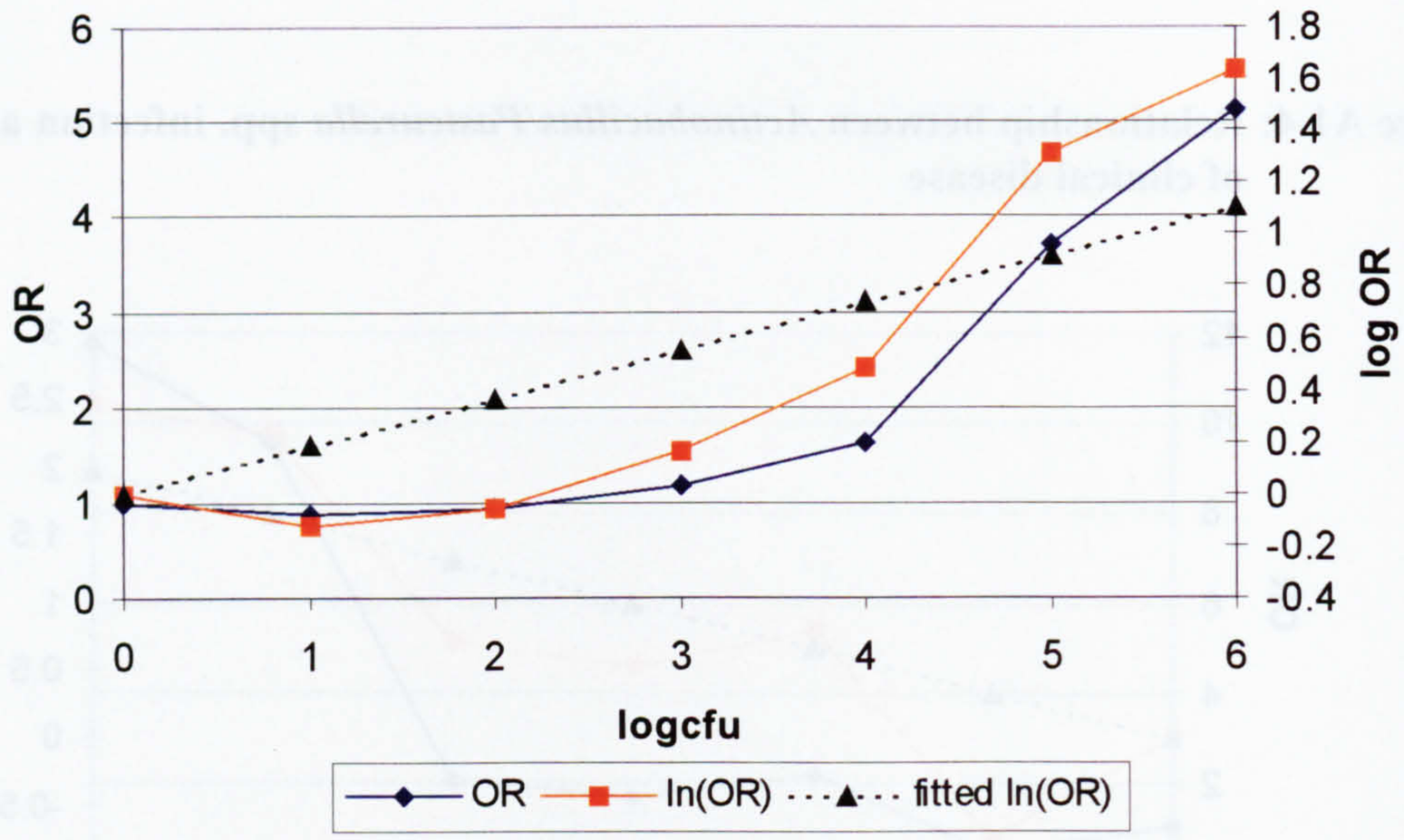


**Figure A1.7: Relationship between *Staphylococcus* spp. infection and odds of clinical disease**

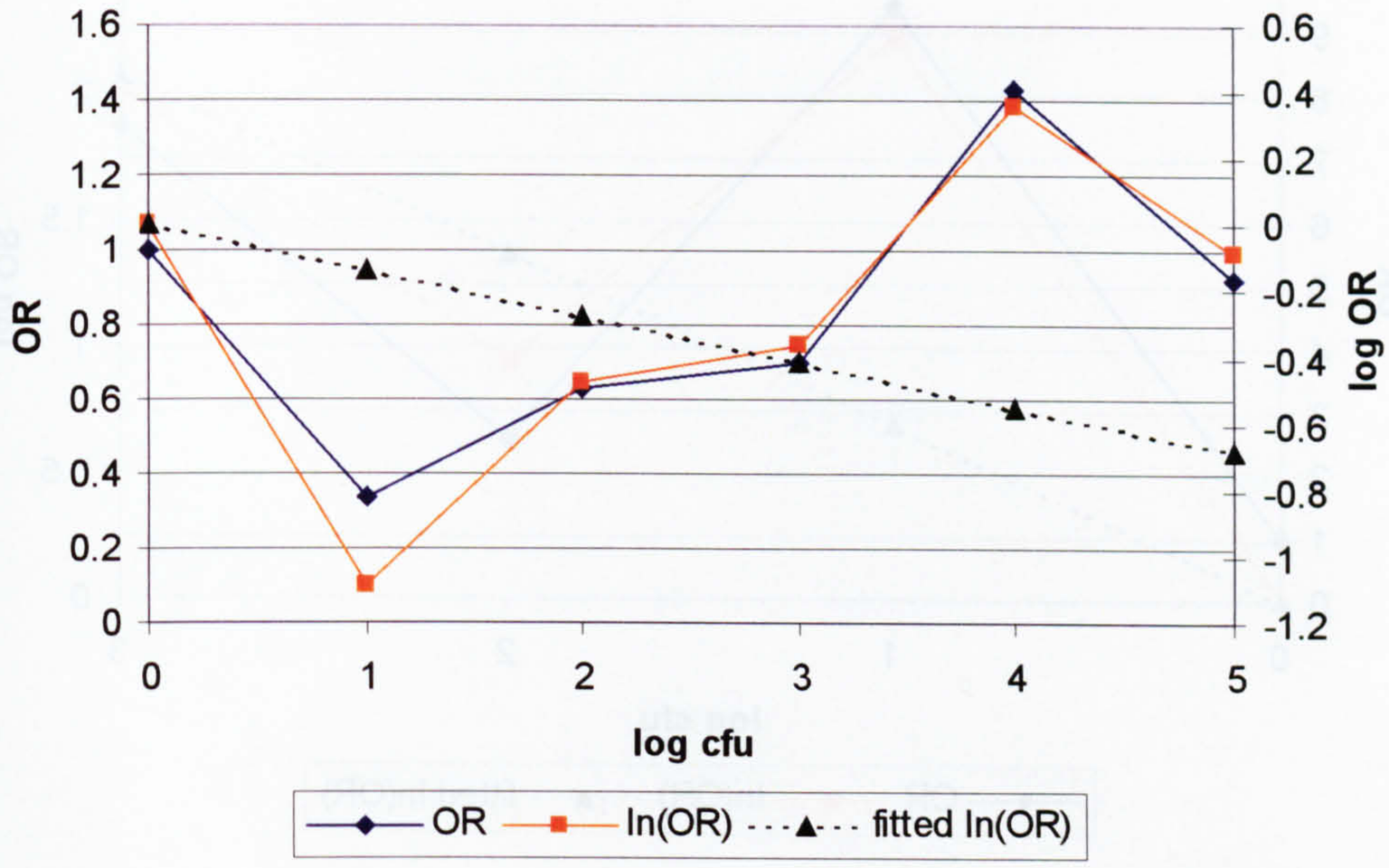



**Figure A1.8: Relationship between *Acinetobacter* spp. infection and odds of clinical disease**

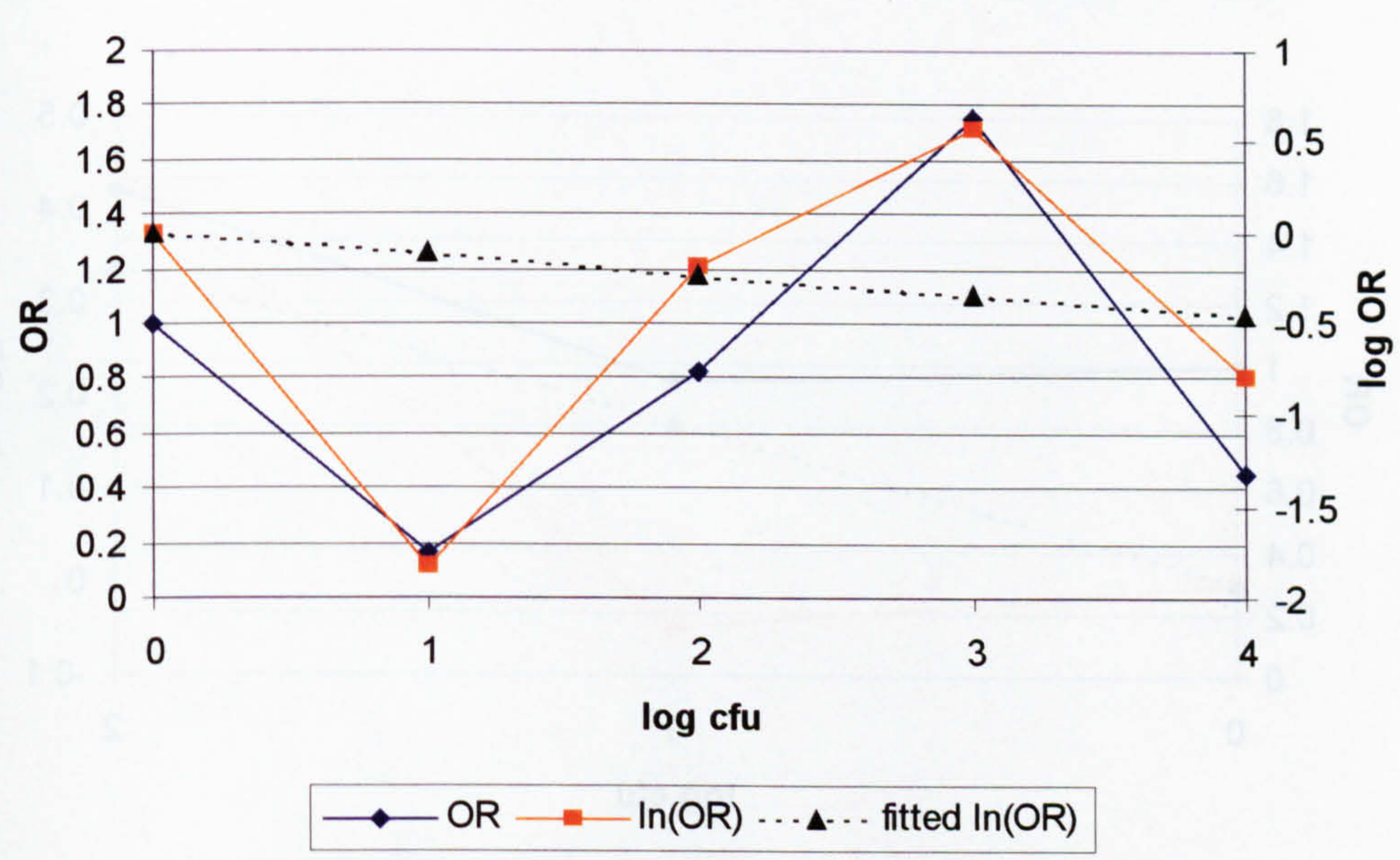


**Figure A1.9: Relationship between *Mycoplasma felis* infection and odds of clinical disease**

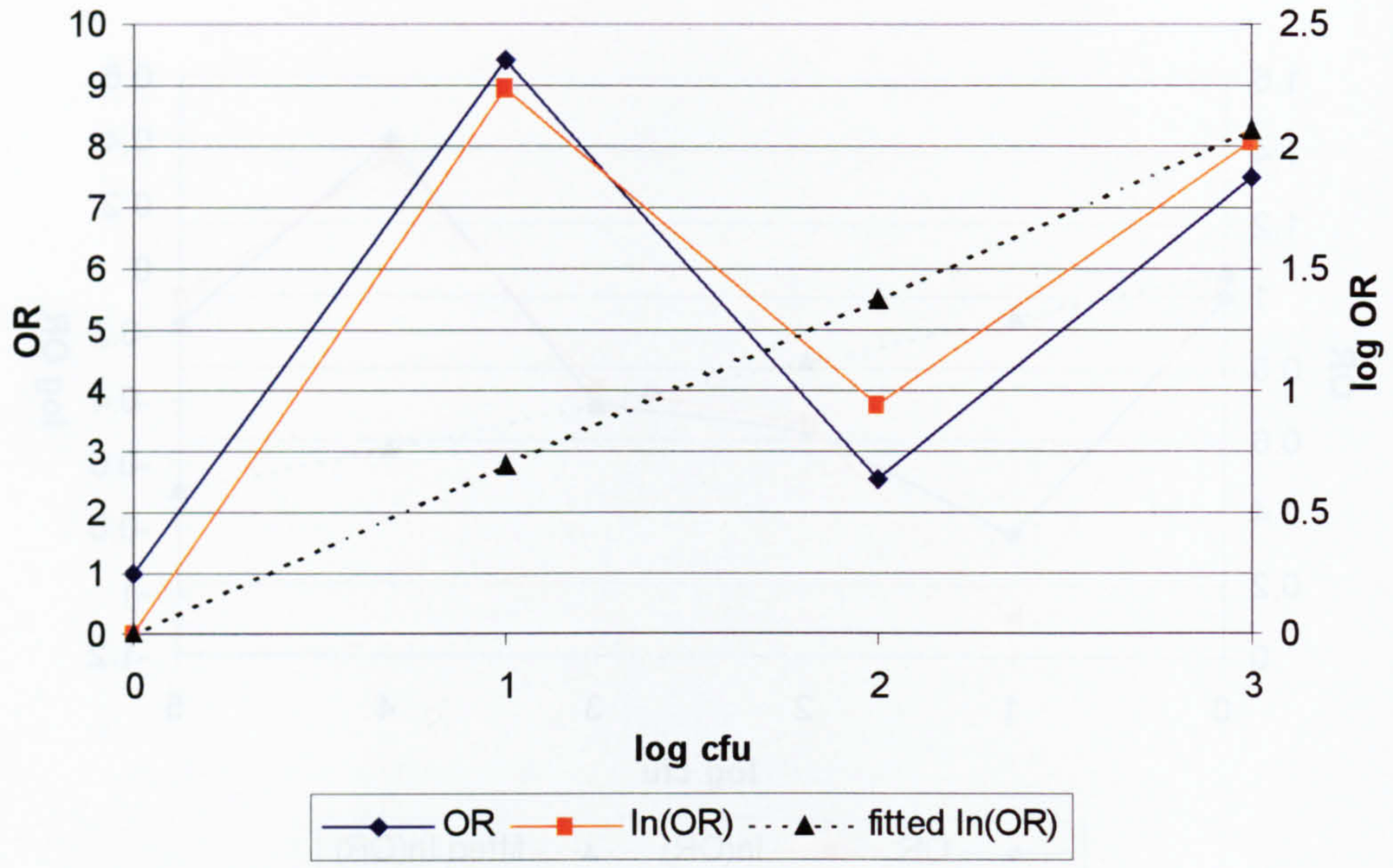
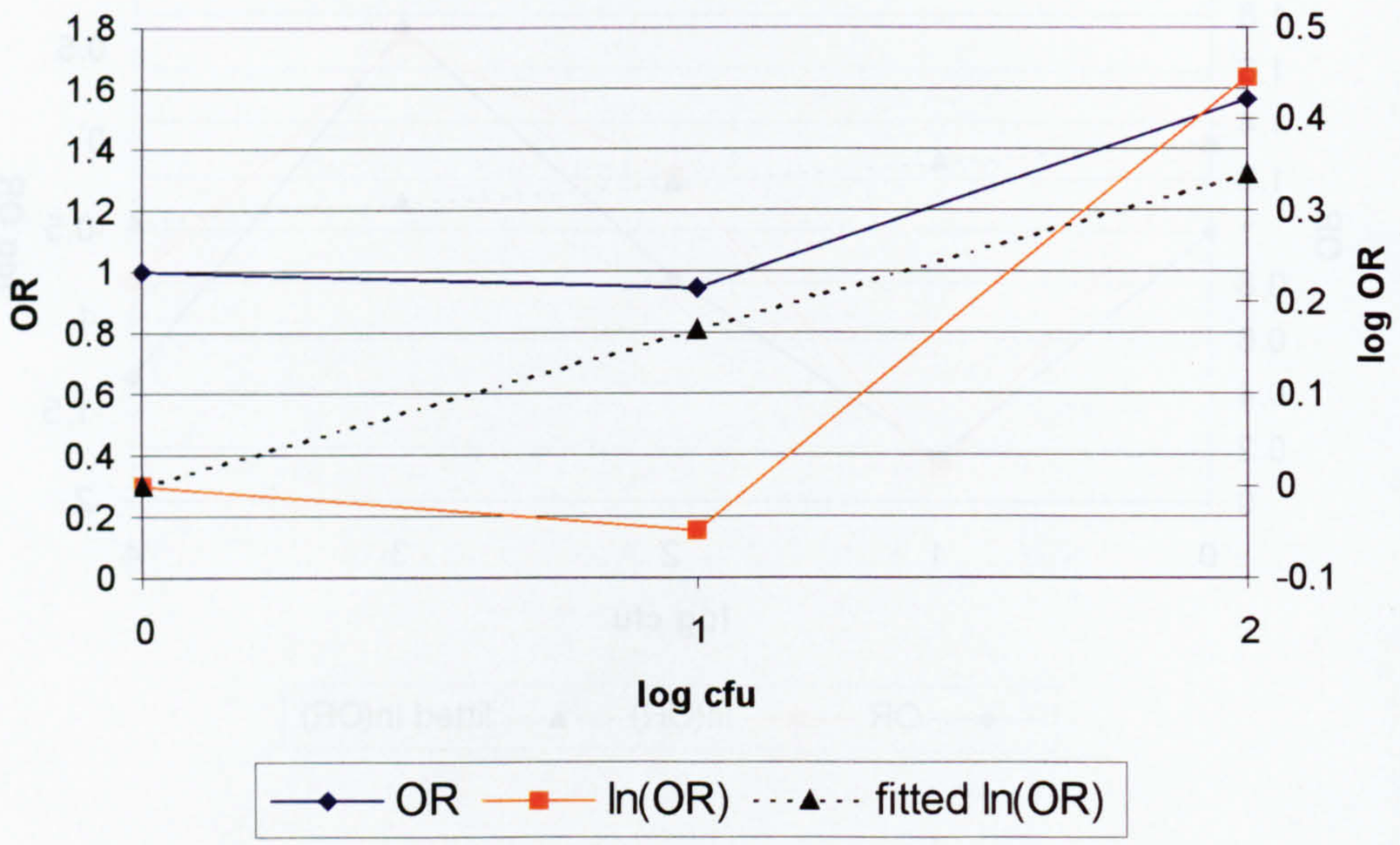



**Figure A1.10: Relationship between *Mycoplasma equirhinis* infection and odds of clinical disease**



# APPENDIX 2

## Chapter 8: Tables & figures

## Table A2.1: Details of pony level explanatory variables and cumulative summary outcome measures

| Pony | Vaccine group | Sex | Transferrin D/F2 | Nasal disch. score | Ocular disch. Score | Cough score | Breathing score | SMLN score | CDNS score | Clinical score |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vaccine | M | D | 1 | 4.75 | 0 | 2 | 1 | 4 | 8.75 |
| 2 | Vaccine | M | D | 5.75 | 5.75 | 0 | 0 | 0 | 5.75 | 11.5 |
| 3 | Vaccine | F | F2 | 4.25 | 11.5 | 0 | 3 | 0 | 7.25 | 18.75 |
| 4 | Vaccine | M | D | 0.75 | 7 | 0 | 0 | 0 | 0.75 | 7.75 |
| 5 | Vaccine | M | D | 16 | 12.75 | 0 | 1 | 3.5 | 20.5 | 33.25 |
| 6 | Vaccine | M | D | 3.5 | 4.25 | 1 | 0 | 1 | 5.5 | 9.75 |
| 7 | Vaccine | M | D | 6.5 | 10.75 | 0 | 0 | 0.5 | 7 | 17.75 |
| 8 | Vaccine | F | F2 | 11 | 9.5 | 2 | 8 | 0.5 | 21.5 | 31 |
| 9 | Vaccine | F | D | 9.25 | 11.75 | 0 | 3 | 5.5 | 17.75 | 29.5 |
| 10 | Vaccine | F | DF2 | 11.75 | 3.75 | 3 | 4 | 4.5 | 23.25 | 27 |
| 11 | Vaccine | F | F2 | 11 | 13 | 2 | 13 | 1.5 | 27.5 | 40.5 |
| 12 | Vaccine | F | DF2 | 10 | 5.25 | 7 | 12 | 2 | 31 | 36.25 |
| 13 | Placebo | M | D | 5 | 8 | 3 | 2 | 0.5 | 10.5 | 18.5 |
| 14 | Placebo | F | F2 | 7.5 | 11.75 | 3 | 4 | 0 | 14.5 | 26.25 |
| 15 | Placebo | F | D | 5.5 | 0.75 | 0 | 0 | 1 | 6.5 | 7.25 |
| 16 | Placebo | M | D | 1.75 | 7.25 | 2 | 2 | 0 | 5.75 | 13 |
| 17 | Placebo | F | D | 3 | 9.75 | 0 | 1 | 3 | 7 | 16.75 |
| 18 | Placebo | M | F2 | 8.25 | 15.25 | 2 | 4 | 1.5 | 15.75 | 31 |
| 19 | Placebo | F | D | 6.5 | 13.5 | 2 | 0 | 2.5 | 11 | 24.5 |
| 20 | Placebo | F | D | 8.75 | 11.5 | 1 | 2 | 3 | 14.75 | 26.25 |
| 21 | Placebo | F | DF2 | 11.5 | 12.5 | 4 | 5 | 1.5 | 22 | 34.5 |
| 22 | Placebo | F | D | 7.75 | 8 | 1 | 3 | 0 | 11.75 | 19.75 |
| 23 | Placebo | M | * | 6.5 | 15.5 | 6 | 10 | 7.5 | 30 | 45.5 |
| 24 | Placebo | F | F2 | 10.5 | 12.5 | 10 | 11 | 2 | 33.5 | 46 |
| 25 | Late intro | M | F2 | 14.25 | 7.5 | 0 | 10 | 9 | 33.25 | 40.75 |
| 26 | Late intro | F | F2 | 14.75 | 5.75 | 3 | 11 | 8 | 36.75 | 42.5 |
| 27 | Late intro | M | F2 | 2.75 | 14.5 | 0 | 2 | 3 | 6.75 | 22.25 |
| 28 | Late intro | M | F2 | 8 | 5 | 3 | 1 | 0.5 | 12.5 | 17.5 |
| 29 | Late intro | M | DF2 | 9.5 | 13.75 | 1 | 11 | 7 | 28.5 | 42.25 |
| Mean | | | | 7.67 | 9.41 | 1.93 | 4.31 | 2.41 | 16.29 | 25.73 |
| S.D. | | | | 4.05 | 3.94 | 2.40 | 4.33 | 2.65 | 10.50 | 12.03 |
| S.E. | | | | 0.75 | 0.73 | 0.45 | 0.80 | 0.49 | 1.95 | 2.23 |
| Median | | | | 7.75 | 9.75 | 1 | 3 | 1.5 | 14.5 | 26.25 |

M male F female *OH2 phenotype

**Table A2.2a: Details of cumulative summary measures for *S. zooepidemicus* in tracheal wash samples**

| Pony | Vaccine group | Sex | Transferrin D/F2 | $Log_{10}$ total cfu/ml | Maximum $log_{10}$ cfu/ml | Mean $log_{10}$ cfu/ml | Weeks $\geq 10^3$ cfu/ml | Weeks $\geq 10^4$ cfu/ml | Weeks $\geq 10^5$ cfu/ml | Week 26 $log_{10}$ cfu/ml |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vaccine | M | D | 3.99 | 3.81 | 2.02 | 2 | 0 | 0 | 0 |
| 2 | Vaccine | M | D | 3.29 | 2.81 | 1.62 | 0 | 0 | 0 | 0 |
| 3 | Vaccine | F | F2 | 4.89 | 4.54 | 2.50 | 3 | 2 | 0 | 1.00 |
| 4 | Vaccine | M | D | 4.93 | 4.88 | 2.14 | 2 | 1 | 0 | 2.36 |
| 5 | Vaccine | M | D | 4.86 | 4.70 | 2.75 | 5 | 2 | 0 | 0 |
| 6 | Vaccine | M | D | 6.05 | 5.97 | 3.97 | 10 | 6 | 1 | 4.61 |
| 7 | Vaccine | M | D | 4.22 | 3.69 | 2.72 | 6 | 0 | 0 | 3.04 |
| 8 | Vaccine | F | F2 | 6.14 | 5.99 | 4.21 | 9 | 7 | 2 | 3.81 |
| 9 | Vaccine | F | D | 7.83 | 7.83 | 3.51 | 7 | 4 | 2 | 0 |
| 10 | Vaccine | F | DF2 | 7.37 | 7.12 | 4.35 | 10 | 6 | 1 | 3.28 |
| 11 | Vaccine | F | F2 | 7.24 | 7.03 | 4.16 | 8 | 7 | 4 | 0 |
| 12 | Vaccine | F | DF2 | 8.21 | 8.02 | 4.91 | 8 | 7 | 6 | 1.48 |
| 13 | Placebo | M | D | 3.93 | 3.63 | 2.52 | 2 | 0 | 0 | 2.71 |
| 14 | Placebo | F | F2 | 5.83 | 5.82 | 2.76 | 5 | 2 | 1 | 3.64 |
| 15 | Placebo | F | D | 5.73 | 5.71 | 2.82 | 8 | 2 | 1 | 0 |
| 16 | Placebo | M | D | 5.21 | 4.92 | 2.91 | 6 | 2 | 0 | 2.30 |
| 17 | Placebo | F | D | 4.38 | 3.95 | 2.70 | 5 | 0 | 0 | 1.48 |
| 18 | Placebo | M | F2 | 6.38 | 6.10 | 3.73 | 8 | 4 | 2 | 4.27 |
| 19 | Placebo | F | D | 5.87 | 5.51 | 4.33 | 10 | 9 | 3 | 4.26 |
| 20 | Placebo | F | D | 7.26 | 7.14 | 4.44 | 8 | 5 | 4 | 2.80 |
| 21 | Placebo | F | DF2 | 7.66 | 7.65 | 3.64 | 3 | 3 | 3 | . |
| 22 | Placebo | F | D | 4.27 | 4.13 | 2.43 | 4 | 1 | 0 | 2.41 |
| 23 | Placebo | M | * | 4.99 | 4.46 | 3.38 | 7 | 5 | 0 | 4.11 |
| 24 | Placebo | F | F2 | 6.30 | 5.81 | 4.51 | 9 | 7 | 6 | 3.32 |
| 25 | Late intro | M | F2 | 6.38 | 6.34 | 4.13 | 10 | 4 | 2 | 6.34 |
| 26 | Late intro | F | F2 | 7.74 | 7.36 | 5.09 | 7 | 7 | 4 | 5.23 |
| 27 | Late intro | M | F2 | 6.41 | 6.18 | 3.33 | 4 | 3 | 3 | 0 |
| 28 | Late intro | M | F2 | 7.69 | 7.28 | 5.01 | 10 | 6 | 5 | 2.89 |
| 29 | Late intro | M | DF2 | 7.01 | 6.87 | 3.80 | 6 | 5 | 4 | 0 |
| Mean | | | | 5.93 | 5.70 | 3.46 | 6.28 | 3.69 | 1.86 | 2.33 |
| S.D. | | | | 1.38 | 1.44 | 0.96 | 2.88 | 2.69 | 1.96 | 1.87 |
| S.E. | | | | 0.26 | 0.27 | 0.18 | 0.53 | 0.50 | 0.36 | 0.35 |
| Median | | | | 6.05 | 5.82 | 3.51 | 7 | 4 | 1 | 2.56 |

M male F female *OH2 phenotype

## Table A2.2b: Details of cumulative summary measures for *A. equuli* in tracheal wash samples

| Pony | Vaccine group | Sex | Transferrin D/F2 | $Log_{10}$ total cfu/ml | Maximum $log_{10}$ cfu/ml | Mean $log_{10}$ cfu/ml | Weeks $\geq 10^3$ cfu/ml | Weeks $\geq 10^4$ cfu/ml | Weeks $\geq 10^5$ cfu/ml | Week 26 $log_{10}$ cfu/ml |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vaccine | M | D | 3.28 | 3.28 | 0.42 | 1 | 0 | 0 | 0 |
| 2 | Vaccine | M | D | 3.47 | 3.32 | 0.86 | 1 | 0 | 0 | 0 |
| 3 | Vaccine | F | F2 | 2.11 | 2.11 | 0.19 | 0 | 0 | 0 | 0 |
| 4 | Vaccine | M | D | 0.00 | 0.00 | 0.00 | 0 | 0 | 0 | 0 |
| 5 | Vaccine | M | D | 3.49 | 3.46 | 0.52 | 1 | 0 | 0 | 0 |
| 6 | Vaccine | M | D | 2.81 | 2.72 | 0.57 | 0 | 0 | 0 | 0 |
| 7 | Vaccine | M | D | 4.36 | 4.36 | 0.70 | 1 | 1 | 0 | 0 |
| 8 | Vaccine | F | F2 | 3.90 | 3.74 | 0.65 | 2 | 0 | 0 | 0 |
| 9 | Vaccine | F | D | 3.62 | 3.62 | 0.33 | 1 | 0 | 0 | 0 |
| 10 | Vaccine | F | DF2 | 0.95 | 1.00 | 0.09 | 0 | 0 | 0 | 0 |
| 11 | Vaccine | F | F2 | 2.17 | 2.11 | 0.31 | 0 | 0 | 0 | 0 |
| 12 | Vaccine | F | DF2 | 6.67 | 6.67 | 1.20 | 1 | 1 | 1 | 0 |
| 13 | Placebo | M | D | 3.15 | 2.97 | 1.10 | 0 | 0 | 0 | 0 |
| 14 | Placebo | F | F2 | 3.61 | 3.60 | 0.56 | 1 | 0 | 0 | 3.60 |
| 15 | Placebo | F | D | 2.25 | 2.00 | 0.47 | 0 | 0 | 0 | 0 |
| 16 | Placebo | M | D | 1.59 | 1.60 | 0.15 | 0 | 0 | 0 | 0 |
| 17 | Placebo | F | D | 2.40 | 2.26 | 0.46 | 0 | 0 | 0 | 0 |
| 18 | Placebo | M | F2 | 2.88 | 2.85 | 0.50 | 0 | 0 | 0 | 2.85 |
| 19 | Placebo | F | D | 1.95 | 1.85 | 0.29 | 0 | 0 | 0 | 0 |
| 20 | Placebo | F | D | 3.55 | 3.54 | 0.70 | 1 | 0 | 0 | 0 |
| 21 | Placebo | F | DF2 | 1.95 | 1.95 | 0.24 | 0 | 0 | 0 | . |
| 22 | Placebo | F | D | 2.41 | 2.32 | 0.37 | 0 | 0 | 0 | 0 |
| 23 | Placebo | M | * | 5.84 | 5.84 | 0.65 | 1 | 1 | 1 | 0 |
| 24 | Placebo | F | F2 | 3.82 | 3.76 | 0.85 | 1 | 0 | 0 | 0 |
| 25 | Late intro | M | F2 | 1.89 | 1.60 | 0.37 | 0 | 0 | 0 | 0 |
| 26 | Late intro | F | F2 | 2.20 | 2.18 | 0.29 | 0 | 0 | 0 | 0 |
| 27 | Late intro | M | F2 | 2.20 | 2.18 | 0.29 | 0 | 0 | 0 | 0 |
| 28 | Late intro | M | F2 | 0.95 | 1.00 | 0.09 | 0 | 0 | 0 | 0 |
| 29 | Late intro | M | DF2 | 3.12 | 3.12 | 0.28 | 1 | 0 | 0 | 0 |
| Mean | | | | 2.85 | 2.79 | 0.47 | 0.45 | 0.10 | 0.07 | 0.23 |
| S.D. | | | | 1.37 | 1.38 | 0.29 | 0.57 | 0.31 | 0.26 | 0.85 |
| S.E. | | | | 0.25 | 0.26 | 0.05 | 0.11 | 0.06 | 0.05 | 0.16 |
| Median | | | | 2.81 | 2.72 | 0.42 | 0 | 0 | 0 | 0 |

M male F female *OH2 phenotype

**Table A2.2c: Details of cumulative summary measures for *Pasteurella* spp. in tracheal wash samples**

| Pony | Vaccine group | Sex | Transferrin D/F2 | $Log_{10}$ total cfu/ml | Maximum $log_{10}$ cfu/ml | Mean $log_{10}$ cfu/ml | Weeks $\geq 10^3$ cfu/ml | Weeks $\geq 10^4$ cfu/ml | Weeks $\geq 10^5$ cfu/ml | Week 26 $log_{10}$ cfu/ml |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vaccine | M | D | 6.04 | 5.94 | 3.17 | 7 | 3 | 2 | 4.90 |
| 2 | Vaccine | M | D | 5.05 | 4.71 | 2.68 | 6 | 3 | 0 | 0 |
| 3 | Vaccine | F | F2 | 5.56 | 5.36 | 2.40 | 5 | 3 | 1 | 2.70 |
| 4 | Vaccine | M | D | 5.56 | 5.30 | 2.88 | 6 | 4 | 2 | 0 |
| 5 | Vaccine | M | D | 5.65 | 5.28 | 1.93 | 4 | 3 | 2 | 0 |
| 6 | Vaccine | M | D | 6.37 | 5.86 | 3.00 | 5 | 5 | 4 | 0 |
| 7 | Vaccine | M | D | 6.13 | 5.96 | 3.23 | 7 | 6 | 2 | 2.48 |
| 8 | Vaccine | F | F2 | 6.62 | 6.27 | 3.58 | 7 | 7 | 4 | 0 |
| 9 | Vaccine | F | D | 6.66 | 6.46 | 3.50 | 8 | 5 | 3 | 3.47 |
| 10 | Vaccine | F | DF2 | 5.94 | 5.56 | 3.86 | 9 | 9 | 4 | 5.56 |
| 11 | Vaccine | F | F2 | 6.50 | 6.27 | 3.56 | 7 | 7 | 5 | 0 |
| 12 | Vaccine | F | DF2 | 7.67 | 7.49 | 5.26 | 9 | 9 | 7 | 6.58 |
| 13 | Placebo | M | D | 5.71 | 5.45 | 2.75 | 4 | 3 | 1 | 2.36 |
| 14 | Placebo | F | F2 | 5.33 | 5.18 | 1.98 | 5 | 2 | 1 | 3.60 |
| 15 | Placebo | F | D | 6.02 | 5.92 | 2.72 | 6 | 4 | 2 | 0 |
| 16 | Placebo | M | D | 5.81 | 5.49 | 2.89 | 7 | 4 | 2 | 3.38 |
| 17 | Placebo | F | D | 4.77 | 4.26 | 2.36 | 6 | 3 | 0 | 2.63 |
| 18 | Placebo | M | F2 | 5.59 | 5.43 | 1.93 | 4 | 3 | 1 | 2.85 |
| 19 | Placebo | F | D | 6.66 | 6.52 | 3.14 | 7 | 5 | 4 | 0 |
| 20 | Placebo | F | D | 7.09 | 6.92 | 3.47 | 7 | 7 | 4 | 0 |
| 21 | Placebo | F | DF2 | 6.39 | 6.38 | 2.75 | 4 | 4 | 1 | . |
| 22 | Placebo | F | D | 6.36 | 6.26 | 2.48 | 5 | 4 | 3 | 0 |
| 23 | Placebo | M | * | 6.10 | 5.85 | 1.41 | 2 | 2 | 2 | 5.73 |
| 24 | Placebo | F | F2 | 6.21 | 5.72 | 3.02 | 7 | 4 | 4 | 0 |
| 25 | Late intro | M | F2 | 7.06 | 6.71 | 4.32 | 9 | 8 | 6 | 5.38 |
| 26 | Late intro | F | F2 | 6.75 | 6.48 | 3.43 | 6 | 6 | 3 | 2.43 |
| 27 | Late intro | M | F2 | 6.65 | 6.62 | 3.38 | 7 | 4 | 2 | 3.98 |
| 28 | Late intro | M | F2 | 6.38 | 6.17 | 2.14 | 4 | 3 | 3 | 2.04 |
| 29 | Late intro | M | DF2 | 6.14 | 5.80 | 3.20 | 7 | 5 | 3 | 4.41 |
| Mean | | | | 6.17 | 5.92 | 2.98 | 6.10 | 4.66 | 2.69 | 2.30 |
| S.D. | | | | 0.63 | 0.68 | 0.78 | 1.70 | 1.97 | 1.67 | 2.17 |
| S.E. | | | | 0.12 | 0.13 | 0.15 | 0.32 | 0.37 | 0.31 | 0.40 |
| Median | | | | 6.14 | 5.92 | 3.00 | 6 | 4 | 2 | 2.46 |

M male F female *OH2 phenotype

## Table A2.2d: Details of cumulative summary measures for *Bordetella bronchiseptica*in tracheal wash samples

| Pony | Vaccine group | Sex | Transferrin D/F2 | $Log_{10}$ total cfu/ml | Maximum $log_{10}$ cfu/ml | Mean $log_{10}$ cfu/ml | Weeks $\geq 10^3$ cfu/ml | Weeks $\geq 10^4$ cfu/ml | Weeks $\geq 10^5$ cfu/ml | Week 26 $log_{10}$ cfu/ml |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vaccine | M | D | 6.29 | 6.13 | 1.81 | 3 | 3 | 2 | 0 |
| 2 | Vaccine | M | D | 6.16 | 6.16 | 0.68 | 1 | 1 | 1 | 0 |
| 3 | Vaccine | F | F2 | 6.47 | 6.44 | 1.53 | 3 | 2 | 2 | 0 |
| 4 | Vaccine | M | D | 6.00 | 6.00 | 1.09 | 1 | 0 | 0 | 0 |
| 5 | Vaccine | M | D | 6.44 | 6.25 | 1.62 | 2 | 2 | 2 | 0 |
| 6 | Vaccine | M | D | 6.71 | 6.67 | 1.52 | 2 | 2 | 2 | 0 |
| 7 | Vaccine | M | D | 5.60 | 5.60 | 1.46 | 2 | 1 | 1 | 0 |
| 8 | Vaccine | F | F2 | 6.02 | 5.79 | 1.34 | 2 | 2 | 2 | 0 |
| 9 | Vaccine | F | D | 6.95 | 6.76 | 1.61 | 2 | 2 | 2 | 0 |
| 10 | Vaccine | F | DF2 | 0.00 | 0.00 | 0.00 | 0 | 0 | 0 | 0 |
| 11 | Vaccine | F | F2 | 6.64 | 6.61 | 1.21 | 2 | 2 | 2 | 0 |
| 12 | Vaccine | F | DF2 | 6.52 | 6.50 | 1.16 | 2 | 2 | 2 | 0 |
| 13 | Placebo | M | D | 5.62 | 5.60 | 1.58 | 2 | 2 | 1 | 0 |
| 14 | Placebo | F | F2 | 6.57 | 6.57 | 1.33 | 1 | 1 | 1 | 0 |
| 15 | Placebo | F | D | 6.29 | 6.29 | 1.23 | 1 | 1 | 1 | 0 |
| 16 | Placebo | M | D | 5.48 | 5.47 | 1.48 | 4 | 1 | 1 | 0 |
| 17 | Placebo | F | D | 6.97 | 6.97 | 1.48 | 2 | 1 | 1 | 0 |
| 18 | Placebo | M | F2 | 6.97 | 6.97 | 1.48 | 2 | 1 | 1 | 0 |
| 19 | Placebo | F | D | 6.04 | 5.89 | 2.73 | 6 | 4 | 4 | 0 |
| 20 | Placebo | F | D | 6.81 | 6.81 | 1.26 | 2 | 1 | 1 | 0 |
| 21 | Placebo | F | DF2 | 3.10 | 3.10 | 0.39 | 1 | 0 | 0 | . |
| 22 | Placebo | F | D | 5.98 | 5.98 | 1.11 | 2 | 1 | 1 | 0 |
| 23 | Placebo | M | * | 4.91 | 4.80 | 1.18 | 2 | 2 | 0 | 0 |
| 24 | Placebo | F | F2 | 7.11 | 6.92 | 1.72 | 2 | 2 | 2 | 0 |
| 25 | Late intro | M | F2 | 6.81 | 6.81 | 1.71 | 3 | 2 | 1 | 0 |
| 26 | Late intro | F | F2 | 2.96 | 2.81 | 0.65 | 0 | 0 | 0 | 0 |
| 27 | Late intro | M | F2 | 4.66 | 4.57 | 1.04 | 2 | 1 | 0 | 0 |
| 28 | Late intro | M | F2 | 6.34 | 6.11 | 3.42 | 7 | 6 | 4 | 0 |
| 29 | Late intro | M | DF2 | 3.73 | 3.73 | 0.41 | 1 | 0 | 0 | 0 |
| Mean | | | | 5.73 | 5.67 | 1.35 | 2.14 | 1.55 | 1.28 | 0.27 |
| S.D. | | | | 1.56 | 1.55 | 0.65 | 1.48 | 1.27 | 1.07 | 1.04 |
| S.E. | | | | 0.29 | 0.29 | 0.12 | 0.28 | 0.24 | 0.20 | 0.19 |
| Median | | | | 6.29 | 6.13 | 1.34 | 2 | 1 | 1 | 0 |

M male F female *OH2 phenotype

**Table A2.2e: Details of cumulative summary measures for non-haemolytic *Streptococcus* spp. in tracheal wash samples**

| Pony | Vaccine group | Sex | Transferrin D/F2 | $Log_{10}$ total cfu/ml | Maximum $log_{10}$ cfu/ml | Mean $log_{10}$ cfu/ml | Weeks $\geq 10^3$ cfu/ml | Weeks $\geq 10^4$ cfu/ml | Weeks $\geq 10^5$ cfu/ml | Week 26 $log_{10}$ cfu/ml |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vaccine | M | D | 5.69 | 5.39 | 3.51 | 8 | 3 | 2 | 2.11 |
| 2 | Vaccine | M | D | 5.39 | 4.91 | 3.70 | 8 | 3 | 0 | 2.08 |
| 3 | Vaccine | F | F2 | 6.31 | 6.21 | 3.19 | 6 | 6 | 3 | 0 |
| 4 | Vaccine | M | D | 6.00 | 5.90 | 3.58 | 7 | 3 | 2 | 3.30 |
| 5 | Vaccine | M | D | 6.07 | 5.76 | 4.10 | 9 | 8 | 2 | 0 |
| 6 | Vaccine | M | D | 6.08 | 5.63 | 4.62 | 11 | 8 | 5 | 4.61 |
| 7 | Vaccine | M | D | 6.37 | 5.97 | 4.53 | 9 | 7 | 6 | 5.97 |
| 8 | Vaccine | F | F2 | 6.62 | 6.34 | 4.26 | 9 | 8 | 5 | 4.08 |
| 9 | Vaccine | F | D | 5.40 | 5.15 | 2.57 | 4 | 4 | 1 | 0 |
| 10 | Vaccine | F | DF2 | 5.21 | 5.15 | 2.25 | 5 | 1 | 1 | 0 |
| 11 | Vaccine | F | F2 | 5.55 | 5.28 | 2.18 | 5 | 3 | 2 | 0 |
| 12 | Vaccine | F | DF2 | 5.22 | 5.10 | 1.66 | 3 | 2 | 1 | 2.89 |
| 13 | Placebo | M | D | 6.38 | 6.13 | 4.78 | 11 | 9 | 4 | 4.00 |
| 14 | Placebo | F | F2 | 6.01 | 5.67 | 3.88 | 9 | 7 | 4 | 5.00 |
| 15 | Placebo | F | D | 6.11 | 5.83 | 3.95 | 7 | 6 | 4 | 2.18 |
| 16 | Placebo | M | D | 5.43 | 4.96 | 3.82 | 9 | 6 | 0 | 4.04 |
| 17 | Placebo | F | D | 5.78 | 5.39 | 3.57 | 7 | 5 | 2 | 0 |
| 18 | Placebo | M | F2 | 5.25 | 5.00 | 2.69 | 7 | 3 | 1 | 0 |
| 19 | Placebo | F | D | 5.48 | 5.14 | 3.58 | 9 | 4 | 1 | 3.26 |
| 20 | Placebo | F | D | 5.41 | 4.86 | 2.72 | 7 | 5 | 0 | 4.84 |
| 21 | Placebo | F | DF2 | 4.03 | 3.99 | 0.97 | 2 | 0 | 0 | . |
| 22 | Placebo | F | D | 5.85 | 5.58 | 4.11 | 9 | 5 | 3 | 3.85 |
| 23 | Placebo | M | * | 7.58 | 7.26 | 5.68 | 11 | 11 | 9 | 5.04 |
| 24 | Placebo | F | F2 | 6.09 | 5.72 | 3.00 | 6 | 3 | 3 | 5.54 |
| 25 | Late intro | M | F2 | 5.94 | 5.52 | 3.49 | 7 | 6 | 2 | 0 |
| 26 | Late intro | F | F2 | 6.64 | 6.64 | 1.29 | 1 | 1 | 1 | 0 |
| 27 | Late intro | M | F2 | 4.89 | 4.83 | 2.54 | 3 | 1 | 0 | 3.14 |
| 28 | Late intro | M | F2 | 5.26 | 5.13 | 2.11 | 5 | 2 | 1 | 2.59 |
| 29 | Late intro | M | DF2 | 2.80 | 2.65 | 0.54 | 0 | 0 | 0 | 1.00 |
| Mean | | | | 5.68 | 5.42 | 3.20 | 6.69 | 4.48 | 2.24 | 2.40 |
| S.D. | | | | 0.86 | 0.83 | 1.20 | 2.93 | 2.84 | 2.13 | 2.07 |
| S.E. | | | | 0.16 | 0.15 | 0.22 | 0.54 | 0.53 | 0.40 | 0.38 |
| Median | | | | 5.78 | 5.39 | 3.51 | 7 | 4 | 2 | 2.59 |

M male F female *OH2 phenotype

# Table A2.3: Summary of results of non-parametric analyses examining differences in cumulative clinical outcome scores according to different pony level explanatory variables

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Clinical score** | Sex | Wilcoxon rank sum | 0.18 |
| | Vaccine group | Kruskal-Wallis | 0.33 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.014** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0024** |
| | Transferrin H1 | Wilcoxon rank sum | 0.72 |
| | Transferrin H2 | Wilcoxon rank sum | 0.85 |
| | Transferrin O | Wilcoxon rank sum | 0.72 |
| | Transferrin R | Wilcoxon rank sum | 0.97 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.38 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.10 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.15 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.40 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.24 |
| **CDNS score** | Sex | Wilcoxon rank sum | 0.06 |
| | Vaccine group | Kruskal-Wallis | 0.29 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.0246** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0014** |
| | Transferrin H1 | Wilcoxon rank sum | 0.77 |
| | Transferrin H2 | Wilcoxon rank sum | 0.61 |
| | Transferrin O | Wilcoxon rank sum | 0.59 |
| | Transferrin R | Wilcoxon rank sum | 0.97 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.54 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.15 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.90 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.45 |
| **Nasal discharge** | Sex | Wilcoxon rank sum | 0.06 |
| | Vaccine group | Kruskal-Wallis | 0.46 |
| | **Transferrin D#** | Wilcoxon rank sum | 0.19 |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0052** |
| | Transferrin H1 | Wilcoxon rank sum | 0.57 |
| | Transferrin H2 | Wilcoxon rank sum | 0.31 |
| | Transferrin O | Wilcoxon rank sum | 0.56 |
| | Transferrin R | Wilcoxon rank sum | 0.59 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.58 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.21 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.90 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.64 |
| **Ocular discharge** | Sex | Wilcoxon rank sum | 0.97 |
| | Vaccine group | Kruskal-Wallis | 0.27 |
| | Transferrin D | Wilcoxon rank sum | 0.08 |
| | Transferrin F2 | Wilcoxon rank sum | 0.37 |
| | Transferrin H1 | Wilcoxon rank sum | 0.64 |
| | Transferrin H2 | Wilcoxon rank sum | 0.70 |
| | Transferrin O | Wilcoxon rank sum | 0.18 |
| | Transferrin R | Wilcoxon rank sum | 0.53 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.24 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.19 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.37 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.23 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.09 |

* 18 ponies with transferrin D haplotype ranked significantly LOWER scores

** 14 ponies with transferrin F2 haplotype ranked significantly HIGHER scores

# Excluding 4 DF2 phenotypes produces P=0.0426 with significantly LOWER ranking in haplotype +ve ponies

Table A2.3 continued

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Cough** | Sex | Wilcoxon rank sum | 0.15 |
| | Vaccine group | Kruskal-Wallis | 0.17 |
| | **Transferrin D[##]** | Wilcoxon rank sum | 0.14 |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0225** |
| | Transferrin H1 | Wilcoxon rank sum | 0.34 |
| | Transferrin H2 | Wilcoxon rank sum | 0.84 |
| | Transferrin O | Wilcoxon rank sum | 0.73 |
| | Transferrin R | Wilcoxon rank sum | 0.56 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.15 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.14 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.32 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.32 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.28 |
| **Breathing** | Sex | Wilcoxon rank sum | 0.08 |
| | Vaccine group | Kruskal-Wallis | 0.48 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.0075** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0002** |
| | Transferrin H1 | Wilcoxon rank sum | 0.52 |
| | Transferrin H2 | Wilcoxon rank sum | 0.75 |
| | Transferrin O | Wilcoxon rank sum | 0.23 |
| | Transferrin R | Wilcoxon rank sum | 0.97 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.54 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.11 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.18 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.40 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.21 |
| **Sub-mandibular LN** | Sex | Wilcoxon rank sum | 0.69 |
| | Vaccine group | Kruskal-Wallis | 0.09 |
| | Transferrin D | Wilcoxon rank sum | 0.60 |
| | Transferrin F2 | Wilcoxon rank sum | 0.33 |
| | Transferrin H1 | Wilcoxon rank sum | 0.26 |
| | Transferrin H2 | Wilcoxon rank sum | 0.95 |
| | Transferrin O | Wilcoxon rank sum | 0.91 |
| | Transferrin R | Wilcoxon rank sum | 0.44 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.98 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.77 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.43 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.26 |

* 18 ponies with transferrin D haplotype ranked significantly LOWER scores

** 14 ponies with transferrin F2 haplotype ranked significantly HIGHER scores

[##] Excluding 4 DF2 phenotypes produces P=0.0228 with significantly LOWER ranking in haplotype +ve ponies

**Table A2.4: Summary of D/F2 transferrin haplotypes ordered by cumulative aggregated and individual clinical outcome scores**

| Pony | Clinical score | Transf. D/F2 | Pony | CDNS score | Transf. D/F2 | Pony | Nasal discharge | Transf. D/F2 | Pony | Ocular discharge | Transf. D/F2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 7.25 | D | 4 | 0.75 | D | 4 | 0.75 | D | 15 | 0.75 | D |
| 4 | 7.75 | D | 1 | 4 | D | 1 | 1 | D | 10 | 3.75 | DF2 |
| 1 | 8.75 | D | 6 | 5.5 | D | 16 | 1.75 | D | 6 | 4.25 | D |
| 6 | 9.75 | D | 2 | 5.75 | D | 27 | 2.75 | F2 | 1 | 4.75 | D |
| 2 | 11.5 | D | 16 | 5.75 | D | 17 | 3 | D | 28 | 5 | F2 |
| 16 | 13 | D | 15 | 6.5 | D | 6 | 3.5 | D | 12 | 5.25 | DF2 |
| 17 | 16.75 | D | 27 | 6.75 | F2 | 3 | 4.25 | F2 | 2 | 5.75 | D |
| 28 | 17.5 | F2 | 17 | 7 | D | 13 | 5 | D | 26 | 5.75 | F2 |
| 7 | 17.75 | D | 7 | 7 | D | 15 | 5.5 | D | 4 | 7 | D |
| 13 | 18.5 | D | 3 | 7.25 | F2 | 2 | 5.75 | D | 16 | 7.25 | D |
| 3 | 18.75 | F2 | 13 | 10.5 | D | 7 | 6.5 | D | 25 | 7.5 | F2 |
| 22 | 19.75 | D | 19 | 11 | D | 19 | 6.5 | D | 13 | 8 | D |
| 27 | 22.25 | F2 | 22 | 11.75 | D | 23 | 6.5 | - | 22 | 8 | D |
| 19 | 24.5 | D | 28 | 12.5 | F2 | 14 | 7.5 | F2 | 8 | 9.5 | F2 |
| 14 | 26.25 | F2 | 14 | 14.5 | F2 | 22 | 7.75 | D | 17 | 9.75 | D |
| 20 | 26.25 | D | 20 | 14.75 | D | 28 | 8 | F2 | 7 | 10.75 | D |
| 10 | 27 | DF2 | 18 | 15.75 | DF2 | 18 | 8.25 | DF2 | 20 | 11.5 | D |
| 9 | 29.5 | D | 9 | 17.75 | D | 20 | 8.75 | D | 3 | 11.5 | F2 |
| 8 | 31 | F2 | 5 | 20.5 | D | 9 | 9.25 | D | 9 | 11.75 | D |
| 18 | 31 | DF2 | 8 | 21.5 | F2 | 29 | 9.5 | DF2 | 14 | 11.75 | F2 |
| 5 | 33.25 | D | 21 | 22 | DF2 | 12 | 10 | DF2 | 21 | 12.5 | DF2 |
| 21 | 34.5 | DF2 | 10 | 23.25 | DF2 | 24 | 10.5 | F2 | 24 | 12.5 | F2 |
| 12 | 36.25 | DF2 | 11 | 27.5 | F2 | 8 | 11 | F2 | 5 | 12.75 | D |
| 11 | 40.5 | F2 | 29 | 28.5 | DF2 | 11 | 11 | F2 | 11 | 13 | F2 |
| 25 | 40.75 | F2 | 23 | 30 | - | 21 | 11.5 | DF2 | 19 | 13.5 | D |
| 29 | 42.25 | DF2 | 12 | 31 | DF2 | 10 | 11.75 | DF2 | 29 | 13.75 | DF2 |
| 26 | 42.5 | F2 | 25 | 33.25 | F2 | 25 | 14.25 | F2 | 27 | 14.5 | F2 |
| 23 | 45.5 | - | 24 | 33.5 | F2 | 26 | 14.75 | F2 | 18 | 15.25 | DF2 |
| 24 | 46 | F2 | 26 | 36.75 | F2 | 5 | 16 | D | 23 | 15.5 | - |
| * P = 0.0024<br># P = 0.002 | | | * P = 0.0014<br># P = 0.0026 | | | * P = 0.0052<br># P = 0.0426 | | | * P = 0.37<br># P = 0.08 | | |

* Wilcoxon rank sum test for transferrin F2 haplotype (highlighted) # Wilcoxon rank sum test for transferrin D haplotype excluding DF2 phenotypes

Table A2.4 continued

| Pony | Cough score | Transf. D/F2 | Pony | Breathing score | Transf. D/F2 | Pony | SMLN score | Transf. D/F2 |
|---|---|---|---|---|---|---|---|---|
| 4 | 0 | D | 4 | 0 | D | 2 | 0 | D |
| 1 | 0 | D | 15 | 0 | D | 4 | 0 | D |
| 17 | 0 | D | 2 | 0 | D | 16 | 0 | D |
| 15 | 0 | D | 7 | 0 | D | 22 | 0 | D |
| 2 | 0 | D | 6 | 0 | D | 3 | 0 | F2 |
| 7 | 0 | D | 19 | 0 | D | 14 | 0 | F2 |
| 9 | 0 | D | 17 | 1 | D | 13 | 0.5 | D |
| 5 | 0 | D | 5 | 1 | D | 7 | 0.5 | D |
| 27 | 0 | F2 | 28 | 1 | F2 | 28 | 0.5 | F2 |
| 3 | 0 | F2 | 1 | 2 | D | 8 | 0.5 | F2 |
| 25 | 0 | F2 | 20 | 2 | D | 15 | 1 | D |
| 6 | 1 | D | 16 | 2 | D | 6 | 1 | D |
| 22 | 1 | D | 13 | 2 | D | 1 | 1 | D |
| 20 | 1 | D | 27 | 2 | F2 | 21 | 1.5 | DF2 |
| 29 | 1 | DF2 | 9 | 3 | D | 18 | 1.5 | DF2 |
| 16 | 2 | D | 22 | 3 | D | 11 | 1.5 | F2 |
| 19 | 2 | D | 3 | 3 | F2 | 12 | 2 | DF2 |
| 18 | 2 | DF2 | 18 | 4 | DF2 | 24 | 2 | F2 |
| 8 | 2 | F2 | 10 | 4 | DF2 | 19 | 2.5 | D |
| 11 | 2 | F2 | 14 | 4 | F2 | 17 | 3 | D |
| 13 | 3 | D | 21 | 5 | DF2 | 20 | 3 | D |
| 10 | 3 | DF2 | 8 | 8 | F2 | 27 | 3 | F2 |
| 14 | 3 | F2 | 25 | 10 | F2 | 5 | 3.5 | D |
| 28 | 3 | F2 | 23 | 10 | - | 10 | 4.5 | DF2 |
| 26 | 3 | F2 | 29 | 11 | DF2 | 9 | 5.5 | D |
| 21 | 4 | DF2 | 26 | 11 | F2 | 29 | 7 | DF2 |
| 23 | 6 | - | 24 | 11 | F2 | 23 | 7.5 | - |
| 12 | 7 | DF2 | 12 | 12 | DF2 | 26 | 8 | F2 |
| 24 | 10 | F2 | 11 | 13 | F2 | 25 | 9 | F2 |
| * P = 0.0225<br># P = 0.0228 | | | * P = 0.0002<br># P = 0.0003 | | | * P = 0.33<br># P = 0.19 | | |

* Wilcoxon rank sum test for transferrin F2 haplotype (highlighted)

# Wilcoxon rank sum test for transferrin D haplotype excluding DF2 phenotype

# Table A2.5: Results of linear regression of cumulative clinical parameter scores and summary explanatory variables for *S. zooepidemicus*

| Outcome variable | Explanatory Variable | Regression coefficient | 95% CI of coefficient | Intercept | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|---|
| **Clinical score** | **$Log_{10}$ total cfu/ml** | **4.7** | **1.8 – 7.6** | **-2.1** | **29.0** | **0.003** |
| | **Maximum $log_{10}$ cfu/ml** | **4.2** | **1.4 – 7.1** | **1.7** | **25.6** | **0.005** |
| | **Geometric mean $log_{10}$ cfu/ml** | **7.6** | **3.7 – 11.6** | **-0.6** | **36.6** | **0.001** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **1.6** | **0.1 – 3.1** | **15.6** | **14.9** | **0.039** |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **2.7** | **1.2 – 4.1** | **16.0** | **35.1** | **0.001** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **3.5** | **1.5 – 5.5** | **19.2** | **32.5** | **0.001** |
| | Week 26 $log_{10}$ cfu/ml | 1.8 | -0.8 – 4.3 | 21.3 | 7.3 | 0.166 |
| **CDNS score** | **$Log_{10}$ total cfu/ml** | **4.5** | **2.1 – 6.9** | **-10.4** | **35.1** | **0.001** |
| | **Maximum $log_{10}$ cfu/ml** | **4.1** | **1.7 – 6.5** | **-7.1** | **31.7** | **0.001** |
| | **Geometric mean $log_{10}$ cfu/ml** | **7.4** | **4.2 – 10.6** | **-9.4** | **45.7** | **<0.001** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **1.7** | **0.4 – 3.0** | **5.7** | **21.3** | **0.012** |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **2.5** | **1.3 – 3.7** | **7.2** | **39.4** | **<0.001** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **3.2** | **1.5 – 4.9** | **10.3** | **36.0** | **0.001** |
| | Week 26 $log_{10}$ cfu/ml | 1.8 | -0.4 – 4.0 | 11.9 | 10.2 | 0.098 |
| **Nasal discharge** | **$Log_{10}$ total cfu/ml** | **6.3** | **2.4 – 10.2** | **-6.8** | **28.8** | **0.003** |
| | **Maximum $log_{10}$ cfu/ml** | **5.9** | **2.2 – 9.7** | **-3.1** | **27.9** | **0.003** |
| | **Geometric mean $log_{10}$ cfu/ml** | **9.8** | **4.3 – 15.2** | **-3.1** | **33.3** | **0.001** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **2.4** | **0.4 – 4.4** | **15.5** | **18.5** | **0.020** |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **3.0** | **0.9 – 5.0** | **19.8** | **24.2** | **0.007** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **3.7** | **0.8 – 6.7** | **23.7** | **17.6** | **0.014** |
| | Week 26 $log_{10}$ cfu/ml | 2.2 | -1.2 – 5.6 | 24.9 | 6.6 | 0.188 |
| Ocular discharge | $Log_{10}$ total cfu/ml | 0.7 | -3.8 – 5.2 | 33.4 | 0.4 | 0.746 |
| | Maximum $log_{10}$ cfu/ml | 0.5 | -3.8 – 4.8 | 35.0 | 0.2 | 0.829 |
| | Geometric mean $log_{10}$ cfu/ml | 0.8 | -5.7 – 7.3 | 34.9 | 0.2 | 0.808 |
| | Total weeks with $\geq 10^3$ cfu/ml | -0.2 | -2.4 – 1.9 | 39.1 | 0.2 | 0.827 |
| | Total weeks with $\geq 10^4$ cfu/ml | 0.8 | -1.5 – 3.1 | 34.6 | 1.9 | 0.475 |
| | Total weeks with $\geq 10^5$ cfu/ml | 1.1 | -2.0 – 4.2 | 35.6 | 1.9 | 0.478 |
| | Week 26 $log_{10}$ cfu/ml | -0.2 | -3.6 – 3.3 | 37.6 | 0.04 | 0.923 |
| **Cough** | **$Log_{10}$ total cfu/ml** | **1.2** | **-0.1– 2.5** | **-3.4** | **12.4** | **0.061** |
| | Maximum $log_{10}$ cfu/ml | 1.0 | -0.2 – 2.3 | -1.9 | 9.1 | 0.111 |
| | **Geometric mean $log_{10}$ cfu/ml** | **2.6** | **0.9 – 4.3** | **-5.0** | **26.1** | **0.005** |
| | Total weeks with $\geq 10^3$ cfu/ml | 0.5 | -0.1 – 1.1 | 0.6 | 9.8 | 0.099 |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **0.9** | **0.4 – 1.5** | **0.5** | **26.1** | **0.005** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **1.4** | **0.6 – 2.2** | **1.3** | **31.3** | **0.002** |
| | Week 26 $log_{10}$ cfu/ml | 0.8 | -0.2 – 1.8 | 1.8 | 10.4 | 0.094 |
| **Breathing** | **$Log_{10}$ total cfu/ml** | **3.1** | **1.0– 5.3** | **-10.0** | **25.0** | **0.006** |
| | **Maximum $log_{10}$ cfu/ml** | **2.9** | **0.8 – 4.9** | **-7.6** | **22.5** | **0.009** |
| | **Geometric mean $log_{10}$ cfu/ml** | **5.0** | **2.0 – 8.0** | **-8.7** | **30.7** | **0.002** |
| | Total weeks with $\geq 10^3$ cfu/ml | 1.0 | -0.2 – 2.1 | 2.5 | 10.4 | 0.088 |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **1.8** | **0.7 – 2.8** | **2.0** | **30.8** | **0.002** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **2.6** | **1.1 – 4.0** | **3.9** | **33.4** | **0.001** |
| | Week 26 $log_{10}$ cfu/ml | 0.9 | -1.0 – 2.8 | 6.5 | 3.5 | 0.338 |
| **SMLN** | **$Log_{10}$ total cfu/ml** | **1.5** | **0.1 – 2.9** | **-4.1** | **15.5** | **0.034** |
| | **Maximum $log_{10}$ cfu/ml** | **1.4** | **0.04 – 2.7** | **-3.1** | **14.3** | **0.043** |
| | **Geometric mean $log_{10}$ cfu/ml** | **2.4** | **0.4 – 4.3** | **-3.4** | **18.3** | **0.021** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **0.6** | **-0.04 – 1.3** | **0.8** | **12.1** | **0.064** |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **0.7** | **-0.01 – 1.4** | **2.2** | **13.2** | **0.053** |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.6 | -0.4 – 1.7 | 3.6 | 5.7 | 0.212 |
| | Week 26 $log_{10}$ cfu/ml | 0.7 | -0.4 – 1.9 | 3.2 | 6.7 | 0.185 |

**Figure A2.1: CDNS score vs. $\log_{10}$ total cfu/ml *S. zooepidemicus* in tracheal washes**

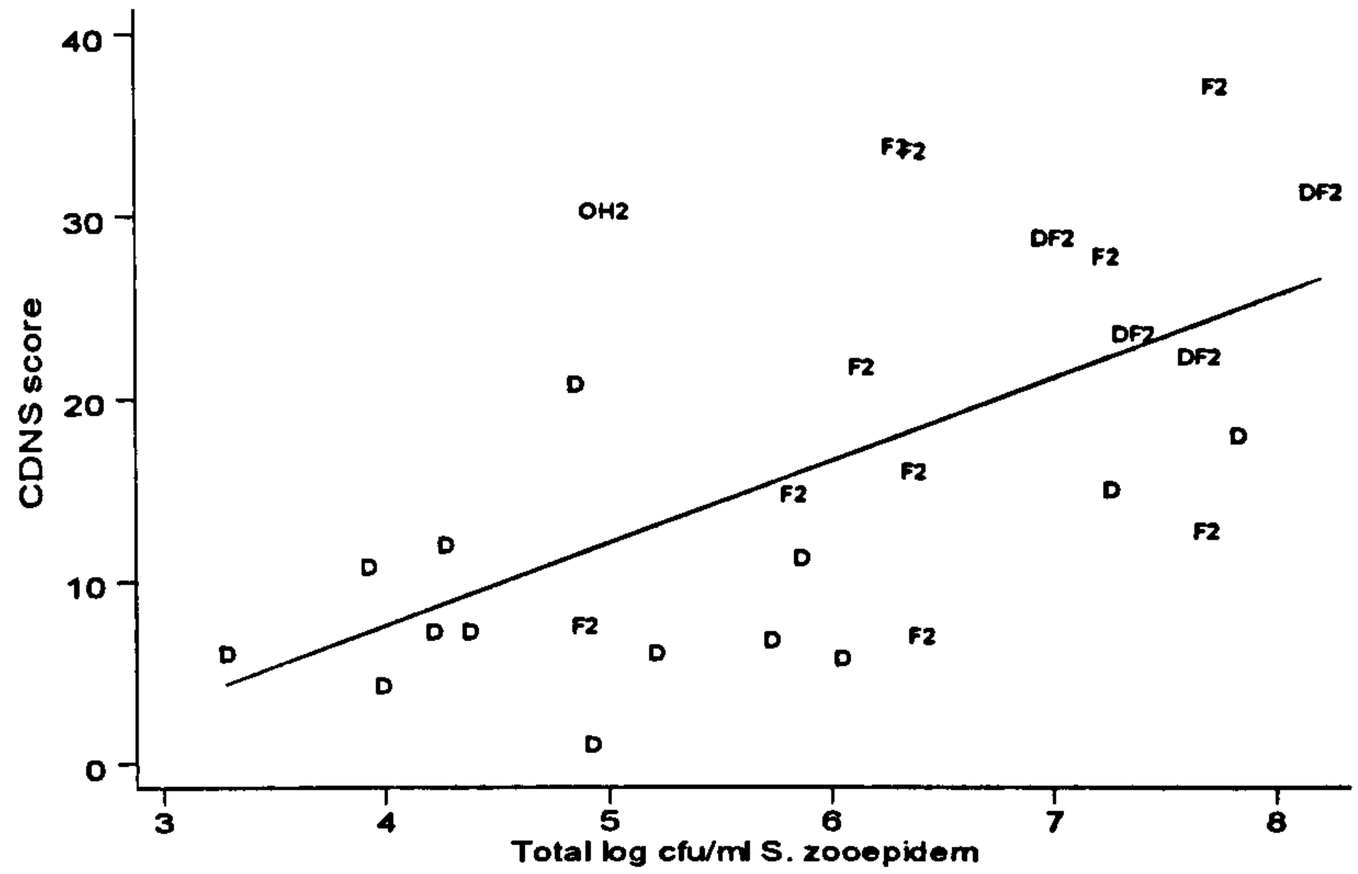



**Figure A2.2: CDNS score vs. maximum $\log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes**

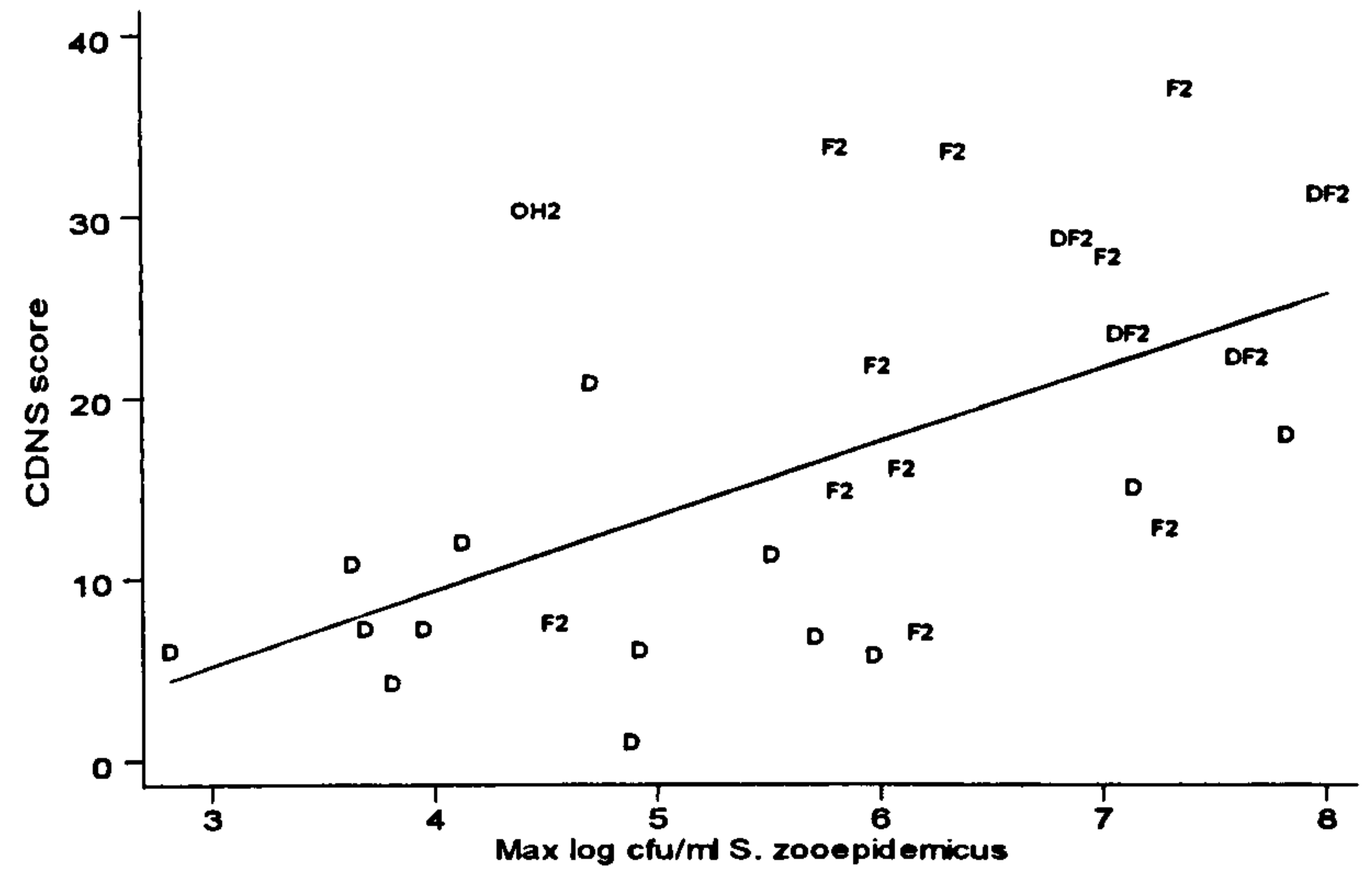



**Figure A2.3: CDNS score vs. mean $\log_{10}$ cfu/ml *S. zooepidemicus* in tracheal washes**

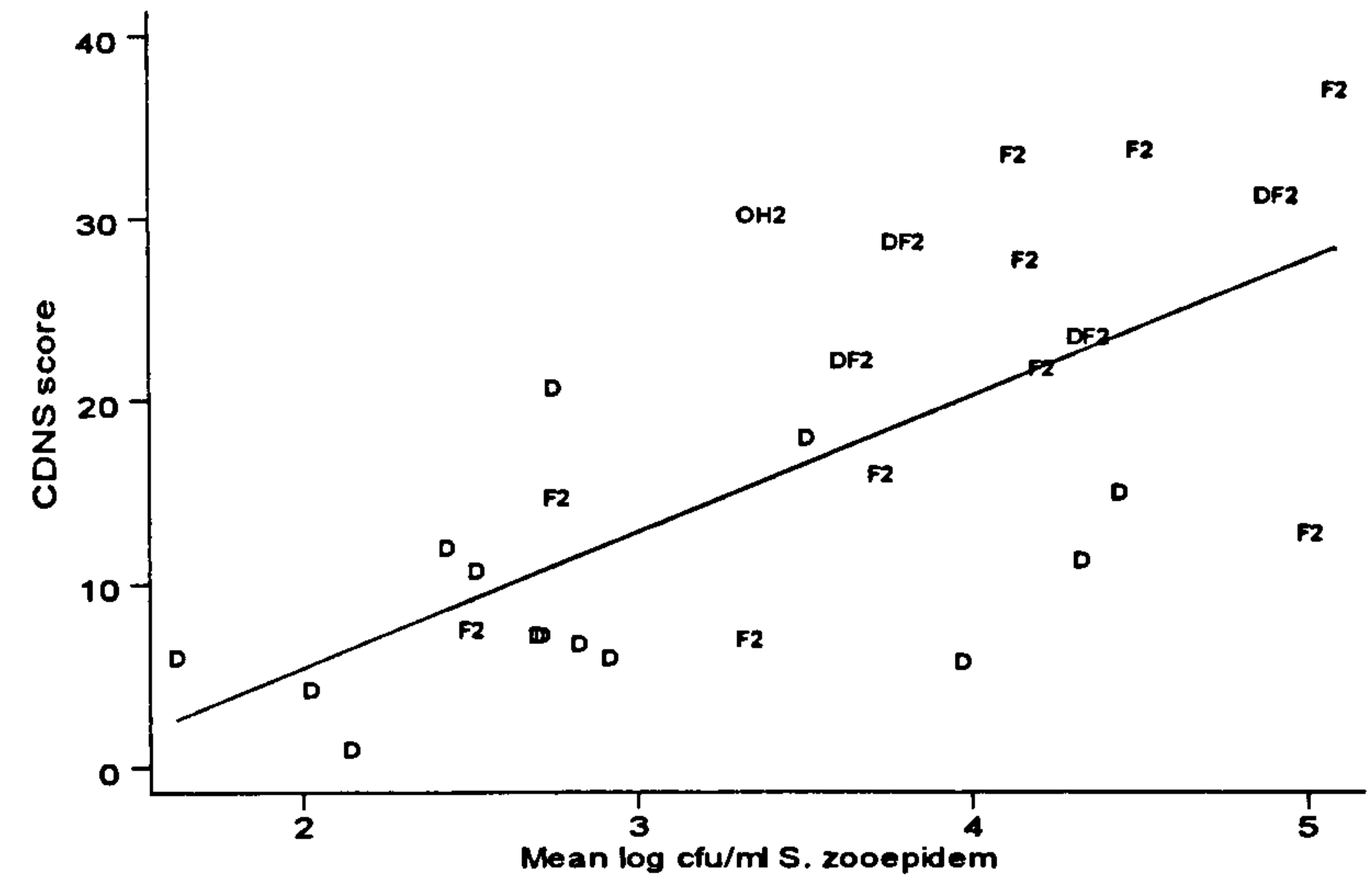


**Figure A2.4: CDNS score vs. total weeks with ≥$10^4$ cfu/ml *S. zooepidemicus* in tracheal washes**

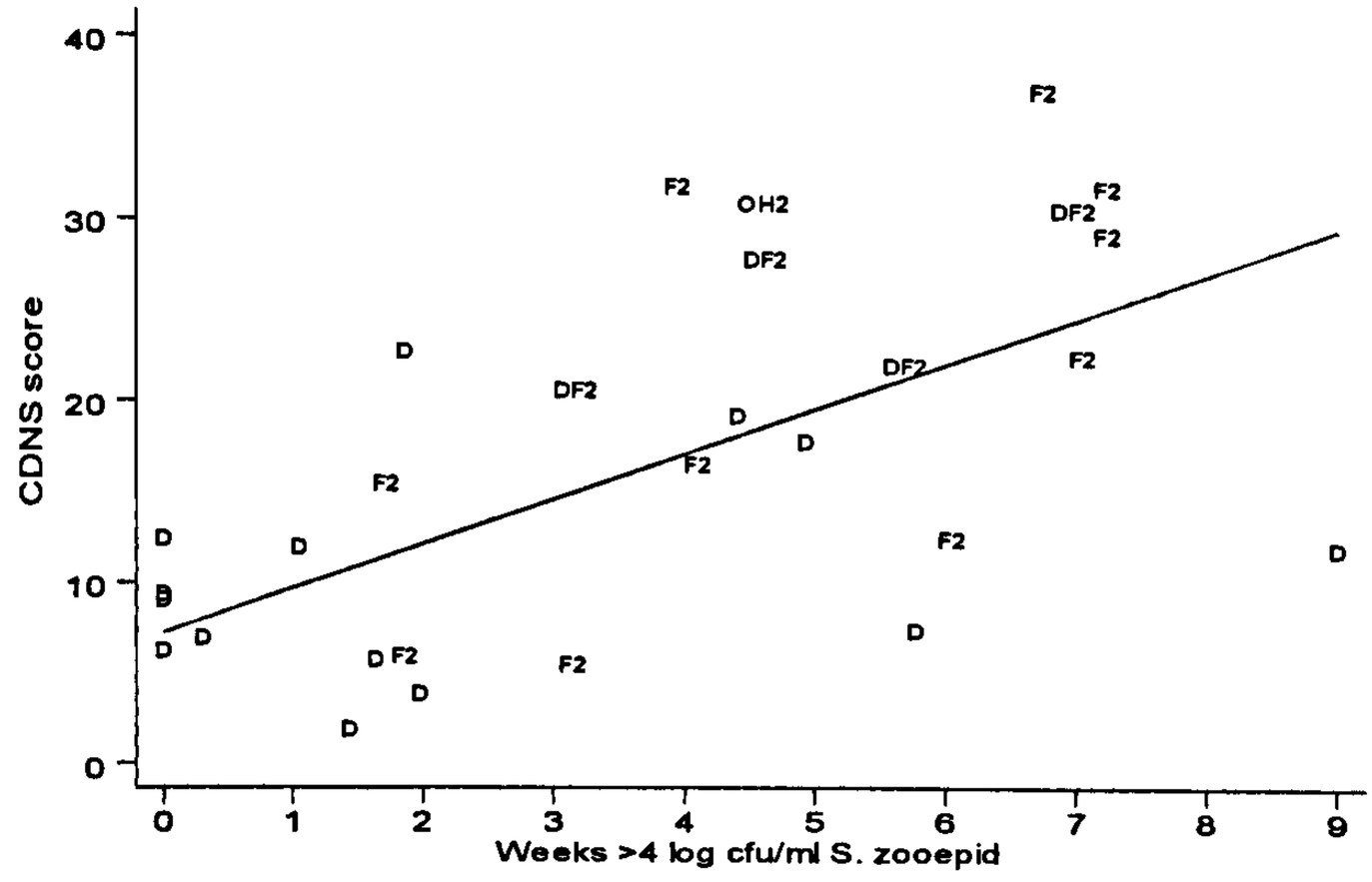
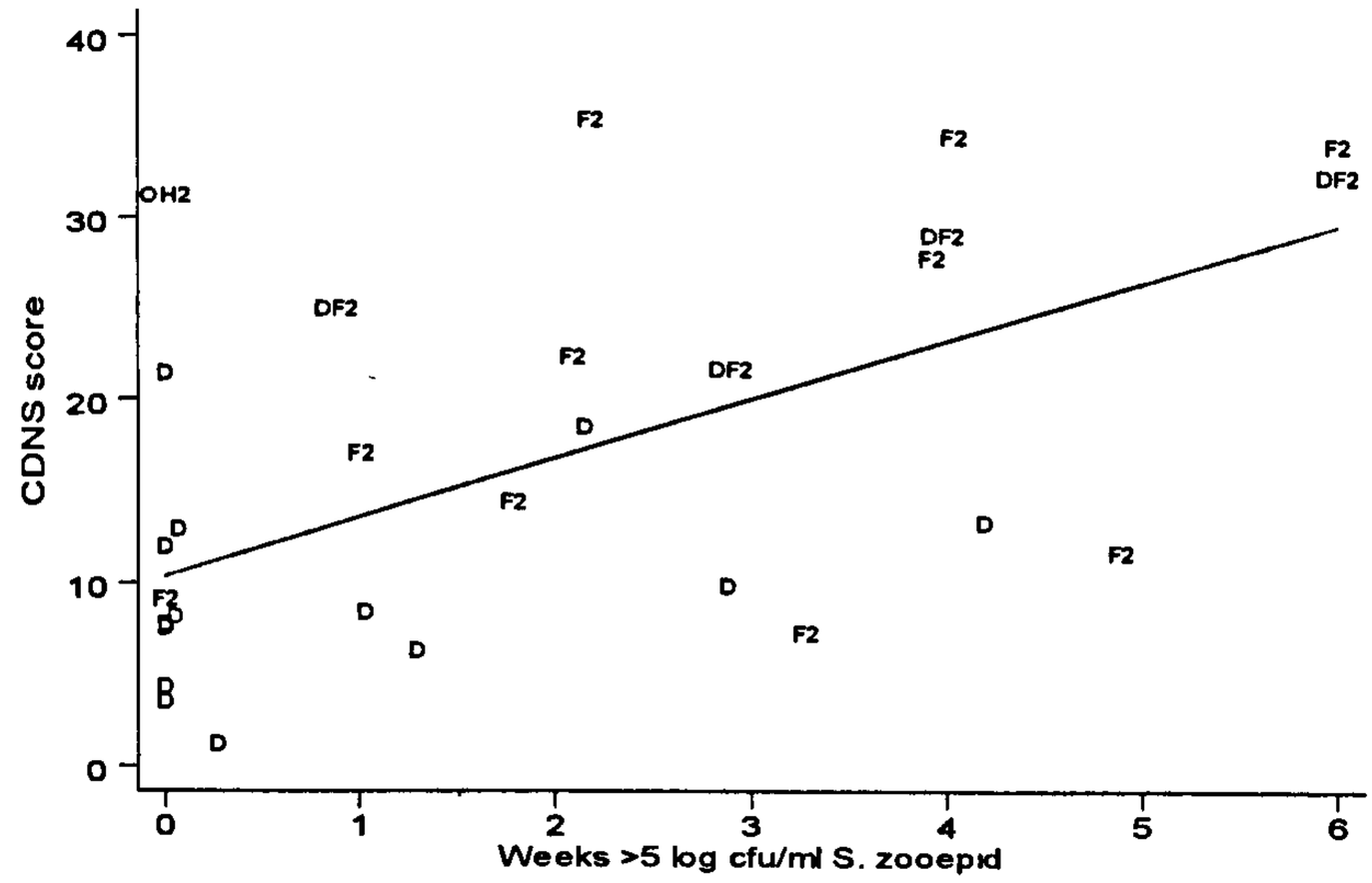



**Figure A2.5: CDNS score vs. total weeks with ≥$10^5$ cfu/ml *S. zooepidemicus* in tracheal washes**



# Table A2.6: Results of linear regression of cumulative clinical parameter scores and summary explanatory variables for *A. equuli*

| Outcome variable | Explanatory Variable | Regression coefficient | 95% CI of coefficient | Intercept | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|---|
| **Clinical score** | **$Log_{10}$ total cfu/ml** | **3.2** | **0.03 – 6.5** | **16.5** | **13.7** | **0.048** |
| | **Maximum $log_{10}$ cfu/ml** | **3.3** | **0.1 – 6.5** | **16.6** | **14.1** | **0.044** |
| | Geometric mean $log_{10}$ cfu/ml | 6.1 | -10.1 – 22.2 | 22.9 | 2.2 | 0.448 |
| | Total weeks with $\geq 10^3$ cfu/ml | 5.5 | -2.5 – 13.5 | 23.2 | 6.9 | 0.168 |
| | Total weeks with $\geq 10^4$ cfu/ml | 8.3 | -6.7 – 23.2 | 24.9 | 4.6 | 0.266 |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **16.3** | **-1.0 – 33.5** | **24.6** | **12.2** | **0.064** |
| | Week 26 $log_{10}$ cfu/ml | 1.0 | -4.8 – 6.7 | 25.2 | 0.5 | 0.732 |
| **CDNS score** | **$Log_{10}$ total cfu/ml** | **2.6** | **-0.3 – 5.4** | **9.0** | **7.4** | **0.074** |
| | **Maximum $log_{10}$ cfu/ml** | **2.6** | **-0.3 – 5.4** | **9.1** | **7.4** | **0.074** |
| | Geometric mean $log_{10}$ cfu/ml | 6.1 | -7.9 – 20.1 | 13.4 | 2.9 | 0.379 |
| | Total weeks with $\geq 10^3$ cfu/ml | 4.2 | -2.9 – 11.2 | 14.4 | 5.2 | 0.233 |
| | Total weeks with $\geq 10^4$ cfu/ml | 7.1 | -6.0 – 20.2 | 15.6 | 4.4 | 0.274 |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **15.3** | **0.4 – 30.2** | **15.2** | **14.1** | **0.045** |
| | Week 26 $log_{10}$ cfu/ml | -0.3 | -5.4 – 4.7 | 16.2 | 0.07 | 0.890 |
| Nasal discharge | $Log_{10}$ total cfu/ml | 1.9 | -2.7 – 6.5 | 25.3 | 2.6 | 0.402 |
| | Maximum $log_{10}$ cfu/ml | 1.9 | -2.7 – 6.4 | 25.5 | 2.5 | 0.413 |
| | Geometric mean $log_{10}$ cfu/ml | 4.8 | -17.1 – 26.7 | 28.5 | 0.7 | 0.659 |
| | Total weeks with $\geq 10^3$ cfu/ml | 5.9 | -5.0 – 16.8 | 28.0 | 4.3 | 0.279 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.03 | -20.7 – 20.6 | 30.7 | 0.00 | 0.998 |
| | Total weeks with $\geq 10^5$ cfu/ml | 2.5 | -22.3 – 27.3 | 30.5 | 0.2 | 0.839 |
| | Week 26 $log_{10}$ cfu/ml | 0.4 | -7.3 – 8.1 | 30.1 | 0.04 | 0.918 |
| Ocular discharge | $Log_{10}$ total cfu/ml | 2.7 | -1.7 – 7.2 | 29.8 | 5.7 | 0.211 |
| | Maximum $log_{10}$ cfu/ml | 2.9 | -1.5 – 7.3 | 29.5 | 6.5 | 0.183 |
| | Geometric mean $log_{10}$ cfu/ml | 0.02 | -21.4 – 21.4 | 37.6 | 0.00 | 0.998 |
| | Total weeks with $\geq 10^3$ cfu/ml | 5.6 | -5.1 – 16.2 | 35.1 | 4.1 | 0.294 |
| | Total weeks with $\geq 10^4$ cfu/ml | 4.9 | -15.1 – 24.9 | 37.1 | 0.9 | 0.620 |
| | Total weeks with $\geq 10^5$ cfu/ml | 4.2 | -19.9 – 28.2 | 37.3 | 0.5 | 0.725 |
| | Week 26 $log_{10}$ cfu/ml | 5.3 | -1.9 – 12.5 | 36.0 | 8.0 | 0.144 |
| **Cough** | **$Log_{10}$ total cfu/ml** | **1.4** | **0.2 – 2.7** | **-0.2** | **16.7** | **0.028** |
| | **Maximum $log_{10}$ cfu/ml** | **1.5** | **0.2 – 2.7** | **-0.2** | **17.7** | **0.023** |
| | **Geometric mean $log_{10}$ cfu/ml** | **6.6** | **0.6 – 12.6** | **0.8** | **16.1** | **0.031** |
| | Total weeks with $\geq 10^3$ cfu/ml | 1.5 | -1.8 – 4.8 | 3.2 | 3.2 | 0.353 |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **5.4** | **-0.4 – 11.1** | **3.3** | **11.9** | **0.066** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **9.8** | **3.6 – 16.1** | **3.2** | **27.7** | **0.003** |
| | Week 26 $log_{10}$ cfu/ml | 0.5 | -1.8 – 2.7 | 3.6 | 0.7 | 0.680 |
| **Breathing** | **$Log_{10}$ total cfu/ml** | **2.3** | **-0.1 – 4.6** | **2.2** | **12.8** | **0.056** |
| | **Maximum $log_{10}$ cfu/ml** | **2.3** | **-0.1 – 4.6** | **2.3** | **12.9** | **0.056** |
| | Geometric mean $log_{10}$ cfu/ml | 5.2 | -6.3 – 16.8 | 6.2 | 3.1 | 0.360 |
| | Total weeks with $\geq 10^3$ cfu/ml | 3.5 | -2.3 – 9.3 | 7.1 | 5.3 | 0.230 |
| | Total weeks with $\geq 10^4$ cfu/ml | 6.7 | -4.0 – 17.4 | 7.9 | 5.8 | 0.207 |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **14.4** | **2.4 – 26.3** | **7.6** | **18.3** | **0.020** |
| | Week 26 $log_{10}$ cfu/ml | -0.2 | -4.4 – 4.0 | 8.6 | 0.03 | 0.927 |
| SMLN | $Log_{10}$ total cfu/ml | 0.5 | -1.0 – 2.0 | 3.5 | 1.5 | 0.521 |
| | Maximum $log_{10}$ cfu/ml | 0.5 | -1.0 – 2.0 | 3.5 | 1.5 | 0.534 |
| | Geometric mean $log_{10}$ cfu/ml | -2.1 | -9.3 – 5.0 | 5.8 | 1.4 | 0.542 |
| | Total weeks with $\geq 10^3$ cfu/ml | 0.4 | -3.3 – 4.0 | 4.7 | 0.2 | 0.544 |
| | Total weeks with $\geq 10^4$ cfu/ml | 2.1 | -4.6 – 8.7 | 4.6 | 1.4 | 0.535 |
| | Total weeks with $\geq 10^5$ cfu/ml | 5.0 | -2.8 – 12.9 | 4.5 | 6.0 | 0.201 |
| | Week 26 $log_{10}$ cfu/ml | -1.2 | -3.7 – 1.3 | 5.2 | 3.5 | 0.343 |

# Table A2.7: Results of linear regression of cumulative clinical parameter scores and summary explanatory variables for *Pasteurella* spp.

| Outcome variable | Explanatory Variable | Regression coefficient | 95% CI of coefficient | Intercept | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|---|
| **Clinical score** | **$Log_{10}$ total cfu/ml** | **8.1** | **1.3 – 14.9** | **-24.2** | **18.1** | **0.022** |
| | **Maximum $log_{10}$ cfu/ml** | **6.6** | **0.1 – 13.1** | **-13.2** | **13.8** | **0.048** |
| ----- | Geometric mean $log_{10}$ cfu/ml | 2.9 | -3.0 – 8.9 | 17.0 | 3.6 | 0.321 |
| | Total weeks with $\geq 10^3$ cfu/ml | 0.5 | -2.3 – 3.3 | 22.7 | 0.5 | 0.715 |
| | Total weeks with $\geq 10^4$ cfu/ml | 1.9 | -0.3 – 4.2 | 16.7 | 10.1 | 0.093 |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **3.1** | **0.6 – 5.7** | **17.3** | **19.1** | **0.018** |
| | Week 26 $log_{10}$ cfu/ml | 1.6 | -0.6 – 3.7 | 21.8 | 7.7 | 0.152 |
| **CDNS score** | **$Log_{10}$ total cfu/ml** | **8.2** | **2.5 – 14.0** | **-34.5** | **24.5** | **0.006** |
| | **Maximum $log_{10}$ cfu/ml** | **6.5** | **1.0 – 12.0** | **-22.2** | **17.7** | **0.023** |
| | Geometric mean $log_{10}$ cfu/ml | 4.4 | -0.6 – 9.4 | 3.0 | 11.0 | 0.079 |
| | Total weeks with $\geq 10^3$ cfu/ml | 1.1 | -1.3 – 3.5 | 9.6 | 3.1 | 0.361 |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **2.4** | **0.6 – 4.3** | **5.0** | **20.8** | **0.013** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **3.5** | **1.5 – 5.6** | **6.8** | **31.4** | **0.002** |
| | Week 26 $log_{10}$ cfu/ml | 1.5 | -0.4 – 3.4 | 12.6 | 9.5 | 0.111 |
| **Nasal discharge** | **$Log_{10}$ total cfu/ml** | **10.5** | **1.3 – 19.8** | **-34.1** | **16.7** | **0.028** |
| | **Maximum $log_{10}$ cfu/ml** | **8.5** | **-0.3 – 17.3** | **-19.4** | **12.6** | **0.059** |
| | Geometric mean $log_{10}$ cfu/ml | 5.2 | -2.7 – 13.1 | 15.3 | 6.2 | 0.191 |
| | Total weeks with $\geq 10^3$ cfu/ml | 1.3 | -2.4 – 5.0 | 22.8 | 1.9 | 0.481 |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **3.8** | **1.0 – 6.7** | **12.8** | **21.8** | **0.011** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **4.4** | **1.0 – 7.8** | **18.9** | **20.5** | **0.014** |
| | Week 26 $log_{10}$ cfu/ml | 0.2 | -2.9 – 3.2 | 29.8 | 0.04 | 0.918 |
| Ocular discharge | $Log_{10}$ total cfu/ml | -0.7 | -10.6 – 9.1 | 42.1 | 0.08 | 0.882 |
| | Maximum $log_{10}$ cfu/ml | 0.1 | -9.1 – 9.2 | 37.3 | 0.00 | 0.989 |
| | Geometric mean $log_{10}$ cfu/ml | -6.1 | -13.7 – 1.4 | 55.9 | 9.3 | 0.108 |
| | Total weeks with $\geq 10^3$ cfu/ml | -2.4 | -6.0 – 1.1 | 52.4 | 6.8 | 0.173 |
| | Total weeks with $\geq 10^4$ cfu/ml | -1.9 | -5.0 – 1.1 | 46.7 | 5.9 | 0.205 |
| | Total weeks with $\geq 10^5$ cfu/ml | -1.5 | -5.1 – 2.2 | 41.6 | 2.4 | 0.422 |
| | Week 26 $log_{10}$ cfu/ml | 0.01 | -2.8 – 3.1 | 36.9 | 0.03 | 0.925 |
| Cough | $Log_{10}$ total cfu/ml | 2.0 | -0.9 – 4.9 | -8.8 | 7.2 | 0.159 |
| | Maximum $log_{10}$ cfu/ml | 1.6 | -1.2 – 4.3 | -5.5 | 5.0 | 0.246 |
| | Geometric mean $log_{10}$ cfu/ml | 0.5 | -1.9 – 2.9 | 2.3 | 0.7 | 0.663 |
| | Total weeks with $\geq 10^3$ cfu/ml | -0.2 | -1.3 – 0.9 | 5.2 | 0.6 | 0.692 |
| | Total weeks with $\geq 10^4$ cfu/ml | 0.2 | -0.7 – 1.2 | 2.8 | 0.9 | 0.632 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.9 | -0.1 – 2.0 | 1.4 | 10.4 | 0.081 |
| | Week 26 $log_{10}$ cfu/ml | 0.4 | -0.5 – 1.3 | 2.8 | 3.3 | 0.358 |
| **Breathing** | **$Log_{10}$ total cfu/ml** | **6.3** | **1.5 – 11.1** | **-30.3** | **21.2** | **0.012** |
| | **Maximum $log_{10}$ cfu/ml** | **5.1** | **0.5 – 9.7** | **-20.5** | **15.9** | **0.032** |
| | **Geometric mean $log_{10}$ cfu/ml** | **4.3** | **0.3 – 8.3** | **-4.2** | **15.0** | **0.038** |
| | Total weeks with $\geq 10^3$ cfu/ml | 1.1 | -0.8 – 3.1 | 1.7 | 5.0 | 0.243 |
| | **Total weeks with $\geq 10^4$ cfu/ml** | **1.9** | **0.3 – 3.4** | **-0.1** | **18.0** | **0.022** |
| | **Total weeks with $\geq 10^5$ cfu/ml** | **2.9** | **1.2 – 4.6** | **0.9** | **30.5** | **0.002** |
| | **Week 26 $log_{10}$ cfu/ml** | **1.4** | **-0.1 – 2.9** | **5.3** | **12.0** | **0.071** |
| **SMLN** | **$Log_{10}$ total cfu/ml** | **2.9** | **-0.2 – 6.0** | **-13.3** | **12.3** | **0.062** |
| | Maximum $log_{10}$ cfu/ml | 2.2 | -0.7 – 5.2 | -8.4 | 8.2 | 0.133 |
| | Geometric mean $log_{10}$ cfu/ml | 1.5 | -1.1 – 4.1 | 0.2 | 5.2 | 0.235 |
| | Total weeks with $\geq 10^3$ cfu/ml | 0.6 | -0.6 – 1.8 | 1.0 | 4.0 | 0.296 |
| | Total weeks with $\geq 10^4$ cfu/ml | 0.8 | -0.2 – 1.8 | 0.9 | 9.8 | 0.098 |
| | Total weeks with $\geq 10^5$ cfu/ml | 1.0 | -0.1 – 2.2 | 2.0 | 10.8 | 0.081 |
| | **Week 26 $log_{10}$ cfu/ml** | **1.2** | **0.3 – 2.0** | **2.2** | **21.8** | **0.012** |

# Table A2.8: Results of linear regression of cumulative clinical parameter scores and summary explanatory variables for *B. bronchiseptica*

| Outcome variable | Explanatory Variable | Regression coefficient | 95% CI of coefficient | Intercept | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|---|
| Clinical score | $Log_{10}$ total cfu/ml | -1.5 | -4.5 – 1.5 | 34.4 | 3.8 | 0.312 |
| | Maximum $log_{10}$ cfu/ml | -1.6 | -4.6 – 1.4 | 34.9 | 4.3 | 0.279 |
| | Geometric mean $log_{10}$ cfu/ml | -3.8 | -11.0 – 3.3 | 30.9 | 4.3 | 0.280 |
| | Total weeks with $\geq 10^3$ cfu/ml | -1.5 | -4.6 – 1.7 | 28.9 | 3.4 | 0.340 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.8 | -4.6 – 2.9 | 27.0 | 0.8 | 0.651 |
| | Total weeks with $\geq 10^5$ cfu/ml | -1.5 | -5.9 – 2.9 | 27.7 | 1.8 | 0.490 |
| | Week 26 $log_{10}$ cfu/ml* | - | - | - | - | - |
| CDNS score | $Log_{10}$ total cfu/ml | -1.8 | -4.4 – 0.8 | 26.5 | 7.0 | 0.165 |
| | Maximum $log_{10}$ cfu/ml | -1.9 | -4.5 – 0.7 | 27.0 | 7.7 | 0.144 |
| | Geometric mean $log_{10}$ cfu/ml | -3.9 | -10.1 – 2.3 | 21.6 | 5.9 | 0.206 |
| | Total weeks with $\geq 10^3$ cfu/ml | -1.6 | -4.4 – 1.1 | 19.8 | 5.4 | 0.227 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.7 | -3.9 – 2.6 | 17.3 | 0.7 | 0.677 |
| | Total weeks with $\geq 10^5$ cfu/ml | -1.2 | -5.1 – 2.6 | 17.9 | 1.6 | 0.516 |
| | Week 26 $log_{10}$ cfu/ml* | - | - | - | - | - |
| Nasal discharge | $Log_{10}$ total cfu/ml | -2.5 | -6.5 – 1.5 | 45.0 | 5.7 | 0.210 |
| | Maximum $log_{10}$ cfu/ml | -2.7 | -6.7 – 1.3 | 45.8 | 6.5 | 0.183 |
| | Geometric mean $log_{10}$ cfu/ml | -4.4 | -14.1 – 5.2 | 36.7 | 3.2 | 0.354 |
| | Total weeks with $\geq 10^3$ cfu/ml | -2.3 | -6.5 – 1.9 | 35.6 | 4.4 | 0.276 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.7 | -5.8 – 4.3 | 31.8 | 0.3 | 0.768 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.1 | -5.9 – 6.1 | 30.5 | 0.01 | 0.962 |
| | Week 26 $log_{10}$ cfu/ml* | - | - | - | - | - |
| Ocular discharge | $Log_{10}$ total cfu/ml | 1.2 | -2.8 – 5.2 | 30.8 | 1.4 | 0.542 |
| | Maximum $log_{10}$ cfu/ml | 1.1 | -2.8 – 5.1 | 31.1 | 1.3 | 0.560 |
| | Geometric mean $log_{10}$ cfu/ml | 0.4 | -9.2 – 9.9 | 37.1 | 0.03 | 0.934 |
| | Total weeks with $\geq 10^3$ cfu/ml | 0.6 | -3.6 – 4.8 | 36.4 | 0.3 | 0.773 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.6 | -5.5 – 4.3 | 38.5 | 0.2 | 0.804 |
| | Total weeks with $\geq 10^5$ cfu/ml | -0.9 | -6.7 – 4.9 | 38.8 | 0.4 | 0.751 |
| | Week 26 $log_{10}$ cfu/ml* | - | - | - | - | - |
| Cough | $Log_{10}$ total cfu/ml | -0.4 | -1.6 – 0.8 | 5.9 | 1.4 | 0.545 |
| | Maximum $log_{10}$ cfu/ml | -0.4 | -1.6 – 0.8 | 6.1 | 1.7 | 0.504 |
| | Geometric mean $log_{10}$ cfu/ml | -0.02 | -2.9 – 2.9 | 3.9 | 0.00 | 0.990 |
| | Total weeks with $\geq 10^3$ cfu/ml | -0.02 | -1.3 – 1.3 | 3.9 | 0.01 | 0.970 |
| | Total weeks with $\geq 10^4$ cfu/ml | 0.4 | -1.0 – 1.9 | 3.2 | 1.4 | 0.542 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.3 | -1.5 – 2.1 | 3.5 | 0.4 | 0.744 |
| | Week 26 $log_{10}$ cfu/ml* | - | - | - | - | - |
| Breathing | $Log_{10}$ total cfu/ml | -0.9 | -3.0 – 1.3 | 13.5 | 2.4 | 0.423 |
| | Maximum $log_{10}$ cfu/ml | -0.9 | -3.1 – 1.3 | 13.7 | 2.6 | 0.405 |
| | Geometric mean $log_{10}$ cfu/ml | -3.8 | -8.8 – 1.2 | 13.8 | 8.2 | 0.133 |
| | Total weeks with $\geq 10^3$ cfu/ml | -1.4 | -3.6 – 0.8 | 11.6 | 5.8 | 0.208 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.8 | -3.5 – 1.9 | 9.9 | 1.4 | 0.546 |
| | Total weeks with $\geq 10^5$ cfu/ml | -1.4 | -4.5 – 1.8 | 10.3 | 2.8 | 0.388 |
| | Week 26 $log_{10}$ cfu/ml* | - | - | - | - | - |
| SMLN | $Log_{10}$ total cfu/ml | -1.1 | -2.4 – 0.1 | 11.3 | 11.2 | 0.076 |
| | Maximum $log_{10}$ cfu/ml | -1.2 | -2.4 – 0.1 | 11.5 | 11.8 | 0.068 |
| | Geometric mean $log_{10}$ cfu/ml | -1.8 | -5.0 – 1.3 | 7.3 | 5.1 | 0.241 |
| | Total weeks with $\geq 10^3$ cfu/ml | -0.7 | -2.1 – 0.7 | 6.3 | 3.9 | 0.305 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.6 | -2.3 – 1.0 | 5.8 | 2.4 | 0.421 |
| | Total weeks with $\geq 10^5$ cfu/ml | -0.6 | -3.4 – 0.3 | 6.8 | 9.9 | 0.097 |
| | Week 26 $log_{10}$ cfu/ml* | - | - | - | - | - |

* Analyses not possible as *B. bronchiseptica* was not isolated from any tracheal washes at week 26 of the study

# Table A2.9: Results of linear regression of cumulative clinical parameter scores and summary explanatory variables for non-haemolytic *Streptococcus* spp.

| Outcome variable | Explanatory Variable | Regression coefficient | 95% CI of coefficient | Intercept | $R^2$ value (%) | P-value |
|---|---|---|---|---|---|---|
| **Clinical score** | $Log_{10}$ total cfu/ml | -1.1 | -6.6 – 4.4 | 31.9 | 0.6 | 0.690 |
| | Maximum $log_{10}$ cfu/ml | -0.6 | -6.4 – 5.1 | 29.2 | 0.2 | 0.818 |
| | **Geometric mean $log_{10}$ cfu/ml** | **-3.9** | **-7.5 – -0.2** | **38.1** | **14.8** | **0.039** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **-1.7** | **-3.2 – -0.2** | **36.9** | **16.7** | **0.028** |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.6 | -2.3 – 1.1 | 28.4 | 2.0 | 0.467 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.1 | -2.1 – 2.3 | 25.5 | 0.03 | 0.929 |
| | Week 26 $log_{10}$ cfu/ml | -1.2 | -3.4 – 1.1 | 28.6 | 4.1 | 0.293 |
| **CDNS score** | $Log_{10}$ total cfu/ml | -0.4 | -5.2 – 4.4 | 18.6 | 0.1 | 0.865 |
| | Maximum $log_{10}$ cfu/ml | 0.1 | -4.9 – 5.1 | 15.8 | 0.00 | 0.973 |
| | **Geometric mean $log_{10}$ cfu/ml** | **-3.7** | **-6.8 – -0.5** | **28.0** | **17.3** | **0.025** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **-1.7** | **-2.8 – -0.3** | **26.8** | **19.2** | **0.017** |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.7 | -2.1 – 0.8 | 19.3 | 3.2 | 0.355 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.04 | -1.9 – 2.0 | 16.2 | 0.01 | 0.964 |
| | Week 26 $log_{10}$ cfu/ml | -1.2 | -3.1 – 0.8 | 19.1 | 2.0 | 0.222 |
| **Nasal discharge** | $Log_{10}$ total cfu/ml | -1.2 | -8.7 – 6.2 | 37.7 | 0.4 | 0.737 |
| | Maximum $log_{10}$ cfu/ml | -0.7 | -8.4 – 7.0 | 34.5 | 0.1 | 0.852 |
| | **Geometric mean $log_{10}$ cfu/ml** | **-5.2** | **-10.1 – -0.3** | **47.4** | **14.7** | **0.040** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **-2.0** | **-4.0 – 0.1** | **43.8** | **12.6** | **0.059** |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.8 | -3.1 – 1.4 | 34.4 | 2.1 | 0.450 |
| | Total weeks with $\geq 10^5$ cfu/ml | -0.9 | -3.9 – 2.1 | 32.7 | 1.4 | 0.540 |
| | **Week 26 $log_{10}$ cfu/ml** | **-3.0** | **-5.8 – -0.1** | **37.8** | **14.2** | **0.044** |
| Ocular discharge | $Log_{10}$ total cfu/ml | -2.6 | -9.8 – 4.6 | 52.3 | 2.0 | 0.469 |
| | Maximum $log_{10}$ cfu/ml | -2.8 | -10.2 – 4.6 | 52.8 | 2.2 | 0.444 |
| | Geometric mean $log_{10}$ cfu/ml | -0.8 | -6.0 – 4.4 | 40.2 | 0.4 | 0.749 |
| | Total weeks with $\geq 10^3$ cfu/ml | -0.4 | -2.5 – 1.8 | 40.1 | 0.5 | 0.727 |
| | Total weeks with $\geq 10^4$ cfu/ml | 0.3 | -1.9 – 2.5 | 36.2 | 0.3 | 0.769 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.3 | -2.6 – 3.2 | 37.0 | 0.2 | 0.841 |
| | Week 26 $log_{10}$ cfu/ml | 0.02 | -3.0 – 3.0 | 37.6 | 0.00 | 0.988 |
| Cough | $Log_{10}$ total cfu/ml | 0.6 | -1.6 – 2.8 | 0.5 | 1.1 | 0.587 |
| | Maximum $log_{10}$ cfu/ml | 0.8 | -1.5 – 3.0 | -0.3 | 1.7 | 0.495 |
| | Geometric mean $log_{10}$ cfu/ml | -0.6 | -2.2 – 1.0 | 5.7 | 2.1 | 0.449 |
| | Total weeks with $\geq 10^3$ cfu/ml | -0.2 | -0.8 – 0.5 | 5.1 | 1.2 | 0.571 |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.1 | -0.8 – 0.5 | 4.5 | 0.6 | 0.685 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.4 | -0.5 – 1.3 | 3.0 | 0.7 | 0.376 |
| | Week 26 $log_{10}$ cfu/ml | 0.7 | -0.1 – 1.6 | 2.1 | 9.9 | 0.099 |
| **Breathing** | $Log_{10}$ total cfu/ml | -0.5 | -4.5 – 3.5 | 11.6 | 0.3 | 0.791 |
| | Maximum $log_{10}$ cfu/ml | -0.1 | -4.2 – 4.0 | 9.2 | 0.01 | 0.960 |
| | **Geometric mean $log_{10}$ cfu/ml** | **-2.9** | **-5.6 – -0.3** | **18.0** | **13.4** | **0.029** |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **-1.3** | **-2.4 – -0.3** | **17.4** | **19.9** | **0.015** |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.6 | -1.8 – 0.6 | 11.3 | 3.8 | 0.308 |
| | Total weeks with $\geq 10^5$ cfu/ml | 0.2 | -1.4 – 1.8 | 8.1 | 0.3 | 0.783 |
| | Week 26 $log_{10}$ cfu/ml | -0.7 | -2.3 – 1.0 | 10.2 | 2.5 | 0.412 |
| **SMLN** | $Log_{10}$ total cfu/ml | -0.3 | -2.8 – 2.1 | 6.8 | 3.4 | 0.776 |
| | Maximum $log_{10}$ cfu/ml | -0.2 | -2.7 – 2.3 | 5.9 | 0.1 | 0.870 |
| | Geometric mean $log_{10}$ cfu/ml | -1.2 | -2.9 – 0.5 | 8.7 | 4.0 | 0.154 |
| | **Total weeks with $\geq 10^3$ cfu/ml** | **-0.7** | **-1.4 – -0.03** | **9.5** | **14.7** | **0.040** |
| | Total weeks with $\geq 10^4$ cfu/ml | -0.2 | -0.9 – 0.5 | 5.7 | 1.2 | 0.575 |
| | Total weeks with $\geq 10^5$ cfu/ml | -0.1 | -1.1 – 0.9 | 5.1 | 0.2 | 0.835 |
| | **Week 26 $log_{10}$ cfu/ml** | **-1.0** | **-1.9 – -0.01** | **7.1** | **10.6** | **0.047** |

# Table A2.10a: Summary of non-parametric analyses examining differences in tracheal *S. zooepidemicus* cumulative variables according to pony level explanatory variables

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **$Log_{10}$ total *S. zooepidemicus*** | **Sex** | Wilcoxon rank sum | **0.05** |
| | Vaccine group | Kruskal-Wallis | 0.09 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.0118** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0011** |
| | Transferrin H1 | Wilcoxon rank sum | 0.20 |
| | Transferrin H2 | Wilcoxon rank sum | 0.53 |
| | Transferrin O | Wilcoxon rank sum | 0.16 |
| | Transferrin R | Wilcoxon rank sum | 0.70 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.93 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.67 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.15 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.19 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.64 |
| **Maximum $Log_{10}$ *S. zooepidemicus*** | **Sex** | Wilcoxon rank sum | **0.05** |
| | Vaccine group | Kruskal-Wallis | 0.11 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.0186** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0012** |
| | Transferrin H1 | Wilcoxon rank sum | 0.13 |
| | Transferrin H2 | Wilcoxon rank sum | 0.41 |
| | Transferrin O | Wilcoxon rank sum | 0.17 |
| | Transferrin R | Wilcoxon rank sum | 0.75 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.97 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.77 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.19 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.23 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.60 |
| **Mean $Log_{10}$ *S. zooepidemicus*** | Sex | Wilcoxon rank sum | 0.08 |
| | Vaccine group | Kruskal-Wallis | 0.15 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.016** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.004** |
| | Transferrin H1 | Wilcoxon rank sum | 0.57 |
| | Transferrin H2 | Wilcoxon rank sum | 0.25 |
| | Transferrin O | Wilcoxon rank sum | 0.37 |
| | Transferrin R | Wilcoxon rank sum | 0.90 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.63 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.15 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.86 |
| **Weeks with $\geq 10^3$ *S. zooepidemicus*** | Sex | Wilcoxon rank sum | 0.24 |
| | Vaccine group | Kruskal-Wallis | 0.66 |
| | Transferrin D | Wilcoxon rank sum | 0.12 |
| | Transferrin F2 | Wilcoxon rank sum | 0.12 |
| | Transferrin H1 | Wilcoxon rank sum | 0.35 |
| | Transferrin H2 | Wilcoxon rank sum | 0.37 |
| | Transferrin O | Wilcoxon rank sum | 0.83 |
| | Transferrin R | Wilcoxon rank sum | 0.97 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.48 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.11 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.90 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.15 |
| | Pr. inhibitor S | Wilcoxon rank sum | 1.00 |

* 14 ponies with transferrin D but no F2 haplotype ranked significantly LOWER scores

** 14 ponies with transferrin F2 haplotype ranked significantly HIGHER scores

Table A2.10a continued

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Weeks with ≥$10^4$ *S. zooepidemicus*** | **Sex** | Wilcoxon rank sum | **0.056** |
| | Vaccine group | Kruskal-Wallis | 0.45 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.0093** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0063** |
| | Transferrin H1 | Wilcoxon rank sum | 0.66 |
| | Transferrin H2 | Wilcoxon rank sum | 0.44 |
| | Transferrin O | Wilcoxon rank sum | 0.79 |
| | Transferrin R | Wilcoxon rank sum | 0.73 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.61 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.10 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.18 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.40 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.82 |
| **Weeks with ≥$10^5$ *S. zooepidemicus*** | **Sex** | Wilcoxon rank sum | **0.06** |
| | Vaccine group | Kruskal-Wallis | 0.07 |
| | **Transferrin D*** | Wilcoxon rank sum | **0.0115** |
| | **Transferrin F2**** | Wilcoxon rank sum | **0.0007** |
| | Transferrin H1 | Wilcoxon rank sum | 0.74 |
| | Transferrin H2 | Wilcoxon rank sum | 0.11 |
| | Transferrin O | Wilcoxon rank sum | 0.17 |
| | Transferrin R | Wilcoxon rank sum | 0.95 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.48 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.17 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.24 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.14 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.49 |
| **$Log_{10}$ *S. zooepidemicus* at week 26** | Sex | Wilcoxon rank sum | 0.87 |
| | Vaccine group | Kruskal-Wallis | 0.29 |
| | Transferrin D | Wilcoxon rank sum | 0.07 |
| | Transferrin F2 | Wilcoxon rank sum | 0.27 |
| | Transferrin H1 | Wilcoxon rank sum | 0.97 |
| | Transferrin H2 | Wilcoxon rank sum | 0.35 |
| | Transferrin O | Wilcoxon rank sum | 0.40 |
| | Transferrin R | Wilcoxon rank sum | 0.71 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.11 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.08 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.75 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.83 |

* 14 ponies with transferrin D but no F2 haplotype ranked significantly LOWER scores

** 14 ponies with transferrin F2 haplotype ranked significantly HIGHER scores

## Table A2.10b: Summary of D/F2 transferrin haplotypes ordered by significant summary variables of *S. zooepidemicus* in tracheal washes

| Pony | $Log_{10}$ total cfu/mL | Transf. D/F2 | Pony | Max. $log_{10}$ cfu/mL | Transf. D/F2 | Pony | Mean $log_{10}$ cfu/ml | Transf. D/F2 | Pony | Weeks $\geq 10^4$ cfu/ml | Transf. D/F2 | Pony | Weeks $\geq 10^5$ cfu/ml | Transf. D/F2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 3.29 | D | 2 | 2.81 | D | 2 | 1.62 | D | 2 | 0 | D | 2 | 0 | D |
| 13 | 3.93 | D | 13 | 3.63 | D | 1 | 2.02 | D | 1 | 0 | D | 1 | 0 | D |
| 1 | 3.99 | D | 7 | 3.69 | D | 4 | 2.14 | D | 13 | 0 | D | 13 | 0 | D |
| 7 | 4.22 | D | 1 | 3.81 | D | 22 | 2.43 | D | 17 | 0 | D | 17 | 0 | D |
| 22 | 4.27 | D | 17 | 3.95 | D | 3 | 2.50 | F2 | 7 | 0 | D | 7 | 0 | D |
| 17 | 4.38 | D | 22 | 4.13 | D | 13 | 2.52 | D | 4 | 1 | D | 4 | 0 | D |
| 5 | 4.86 | D | 23 | 4.46 | - | 17 | 2.70 | D | 22 | 1 | D | 22 | 0 | D |
| 3 | 4.89 | F2 | 3 | 4.54 | F2 | 7 | 2.72 | D | 5 | 2 | D | 5 | 0 | D |
| 4 | 4.93 | D | 5 | 4.70 | D | 5 | 2.75 | D | 15 | 2 | D | 16 | 0 | D |
| 23 | 4.99 | - | 4 | 4.88 | D | 14 | 2.76 | F2 | 16 | 2 | D | 3 | 0 | F2 |
| 16 | 5.21 | D | 16 | 4.92 | D | 15 | 2.82 | D | 3 | 2 | F2 | 23 | 0 | - |
| 15 | 5.73 | D | 19 | 5.51 | D | 16 | 2.91 | D | 14 | 2 | F2 | 15 | 1 | D |
| 14 | 5.83 | F2 | 15 | 5.71 | D | 27 | 3.33 | F2 | 21 | 3 | DF2 | 6 | 1 | D |
| 19 | 5.87 | D | 24 | 5.81 | F2 | 23 | 3.38 | - | 27 | 3 | F2 | 10 | 1 | DF2 |
| 6 | 6.05 | D | 14 | 5.82 | F2 | 9 | 3.51 | D | 9 | 4 | D | 14 | 1 | F2 |
| 8 | 6.14 | F2 | 6 | 5.97 | D | 21 | 3.64 | DF2 | 18 | 4 | F2 | 9 | 2 | D |
| 24 | 6.30 | F2 | 8 | 5.99 | F2 | 18 | 3.73 | F2 | 25 | 4 | F2 | 18 | 2 | F2 |
| 18 | 6.38 | F2 | 18 | 6.10 | F2 | 29 | 3.80 | DF2 | 20 | 5 | D | 25 | 2 | F2 |
| 25 | 6.38 | F2 | 27 | 6.18 | F2 | 6 | 3.97 | D | 29 | 5 | DF2 | 8 | 2 | F2 |
| 27 | 6.41 | F2 | 25 | 6.34 | F2 | 25 | 4.13 | F2 | 23 | 5 | - | 19 | 3 | D |
| 29 | 7.01 | DF2 | 29 | 6.87 | DF2 | 11 | 4.16 | F2 | 6 | 6 | D | 21 | 3 | DF2 |
| 11 | 7.24 | F2 | 11 | 7.03 | F2 | 8 | 4.21 | F2 | 10 | 6 | DF2 | 27 | 3 | F2 |
| 20 | 7.26 | D | 10 | 7.12 | DF2 | 19 | 4.33 | D | 28 | 6 | F2 | 20 | 4 | D |
| 10 | 7.37 | DF2 | 20 | 7.14 | D | 10 | 4.35 | DF2 | 12 | 7 | DF2 | 29 | 4 | DF2 |
| 21 | 7.66 | DF2 | 28 | 7.28 | F2 | 20 | 4.44 | D | 11 | 7 | F2 | 11 | 4 | F2 |
| 28 | 7.69 | F2 | 26 | 7.36 | F2 | 24 | 4.51 | F2 | 8 | 7 | F2 | 26 | 4 | F2 |
| 26 | 7.74 | F2 | 21 | 7.65 | DF2 | 12 | 4.91 | DF2 | 24 | 7 | F2 | 28 | 5 | F2 |
| 9 | 7.83 | D | 9 | 7.83 | D | 28 | 5.01 | F2 | 26 | 7 | F2 | 12 | 6 | DF2 |
| 12 | 8.21 | DF2 | 12 | 8.02 | DF2 | 26 | 5.09 | F2 | 19 | 9 | D | 24 | 6 | F2 |
| * P = 0.0011<br># P = 0.0118 | | | * P = 0.0012<br># P = 0.0186 | | | * P = 0.004<br># P = 0.016 | | | * P = 0.0063<br># P = 0.0093 | | | * P = 0.0007<br># P = 0.0115 | | |

* Wilcoxon rank sum test for transferrin F2 haplotype (highlighted)

# Wilcoxon rank sum test for transferrin D haplotype excluding DF2 phenotypes

**Table A2.11: Summary of non-parametric analyses examining differences in tracheal *A. equuli* cumulative variables according to pony level explanatory variables**

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **$Log_{10}$ total *A. Equuli*** | Sex | Wilcoxon rank sum | 0.79 |
| | Vaccine group | Kruskal-Wallis | 0.22 |
| | Transferrin D | Wilcoxon rank sum | 0.95 |
| | Transferrin F2 | Wilcoxon rank sum | 0.38 |
| | Transferrin H1 | Wilcoxon rank sum | 0.67 |
| | Transferrin H2 | Wilcoxon rank sum | 0.31 |
| | Transferrin O | Wilcoxon rank sum | 0.57 |
| | Transferrin R | Wilcoxon rank sum | 0.45 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.52 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.59 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.14 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.47 |
| **Maximum $Log_{10}$ *A. equuli*** | Sex | Wilcoxon rank sum | 0.83 |
| | Vaccine group | Kruskal-Wallis | 0.27 |
| | Transferrin D | Wilcoxon rank sum | 0.93 |
| | Transferrin F2 | Wilcoxon rank sum | 0.53 |
| | Transferrin H1 | Wilcoxon rank sum | 0.59 |
| | Transferrin H2 | Wilcoxon rank sum | 0.30 |
| | Transferrin O | Wilcoxon rank sum | 0.78 |
| | Transferrin R | Wilcoxon rank sum | 0.43 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.44 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.68 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.14 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.53 |
| **Mean $Log_{10}$ *A. equuli*** | Sex | Wilcoxon rank sum | 0.90 |
| | Vaccine group | Kruskal-Wallis | 0.16 |
| | Transferrin D | Wilcoxon rank sum | 0.84 |
| | Transferrin F2 | Wilcoxon rank sum | 0.22 |
| | Transferrin H1 | Wilcoxon rank sum | 0.32 |
| | Transferrin H2 | Wilcoxon rank sum | 0.70 |
| | Transferrin O | Wilcoxon rank sum | 0.67 |
| | Transferrin R | Wilcoxon rank sum | 0.41 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.58 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.13 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.51 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.14 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.44 |
| **Weeks with $\geq 10^3$ *A. equuli*** | Sex | Wilcoxon rank sum | 1.00 |
| | Vaccine group | Kruskal-Wallis | 0.34 |
| | Transferrin D | Wilcoxon rank sum | 0.84 |
| | Transferrin F2 | Wilcoxon rank sum | 0.69 |
| | Transferrin H1 | Wilcoxon rank sum | 0.74 |
| | Transferrin H2 | Wilcoxon rank sum | 0.19 |
| | Transferrin O | Wilcoxon rank sum | 0.66 |
| | Transferrin R | Wilcoxon rank sum | 0.77 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.54 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.18 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.41 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.41 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.28 |

Table A2.11 continued

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Weeks with $\geq 10^4$ *A. equuli*** | Sex | Wilcoxon rank sum | 0.51 |
| | Vaccine group | Kruskal-Wallis | 0.86 |
| | Transferrin D | Wilcoxon rank sum | 0.86 |
| | Transferrin F2 | Wilcoxon rank sum | 0.59 |
| | Transferrin H1 | Wilcoxon rank sum | 0.54 |
| | Transferrin H2 | Wilcoxon rank sum | 0.31 |
| | Transferrin O | Wilcoxon rank sum | 0.93 |
| | Transferrin R | Wilcoxon rank sum | 0.31 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.68 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.54 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.73 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.73 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.41 |
| **Weeks with $\geq 10^5$ *A. equuli*** | Sex | Wilcoxon rank sum | 0.96 |
| | Vaccine group | Kruskal-Wallis | 0.96 |
| | Transferrin D | Wilcoxon rank sum | 0.72 |
| | Transferrin F2 | Wilcoxon rank sum | 0.96 |
| | Transferrin H1 | Wilcoxon rank sum | 0.62 |
| | Transferrin H2 | Wilcoxon rank sum | 0.13 |
| | Transferrin O | Wilcoxon rank sum | 0.55 |
| | Transferrin R | Wilcoxon rank sum | 0.56 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.88 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.62 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.79 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.79 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.51 |
| **$Log_{10}$ *A. equuli* at week 26** | Sex | Wilcoxon rank sum | 0.96 |
| | Vaccine group | Kruskal-Wallis | 0.73 |
| | Transferrin D | Wilcoxon rank sum | 0.07 |
| | Transferrin F2 | Wilcoxon rank sum | 0.12 |
| | Transferrin H1 | Wilcoxon rank sum | 0.62 |
| | Transferrin H2 | Wilcoxon rank sum | 0.56 |
| | Transferrin O | Wilcoxon rank sum | 0.04 |
| | Transferrin R | Wilcoxon rank sum | 0.56 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.10 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.08 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.78 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.78 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.25 |

## Table A2.12: Summary of non-parametric analyses examining differences in tracheal *Pasteurella* spp. cumulative variables according to pony level explanatory variables

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **$Log_{10}$ total *Pasteurella* spp.** | Sex | Wilcoxon rank sum | 0.16 |
| | Vaccine group | Kruskal-Wallis | 0.12 |
| | Transferrin D | Wilcoxon rank sum | 0.50 |
| | Transferrin F2 | Wilcoxon rank sum | 0.18 |
| | Transferrin H1 | Wilcoxon rank sum | 0.22 |
| | Transferrin H2 | Wilcoxon rank sum | 0.49 |
| | **Transferrin O*** | Wilcoxon rank sum | **0.04** |
| | Transferrin R | Wilcoxon rank sum | 0.21 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.10 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.94 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.19 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.63 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.45 |
| **Maximum $Log_{10}$ *Pasteurella* spp.** | Sex | Wilcoxon rank sum | 0.16 |
| | Vaccine group | Kruskal-Wallis | 0.18 |
| | Transferrin D | Wilcoxon rank sum | 0.62 |
| | Transferrin F2 | Wilcoxon rank sum | 0.26 |
| | Transferrin H1 | Wilcoxon rank sum | 0.15 |
| | Transferrin H2 | Wilcoxon rank sum | 0.41 |
| | **Transferrin O*** | Wilcoxon rank sum | **0.05** |
| | Transferrin R | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.04 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.64 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.28 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.72 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.36 |
| **Mean $Log_{10}$ *Pasteurella* spp.** | Sex | Wilcoxon rank sum | 0.18 |
| | Vaccine group | Kruskal-Wallis | 0.05 |
| | Transferrin D | Wilcoxon rank sum | 0.75 |
| | Transferrin F2 | Wilcoxon rank sum | 0.14 |
| | Transferrin H1 | Wilcoxon rank sum | 0.43 |
| | Transferrin H2 | Wilcoxon rank sum | 0.45 |
| | **Transferrin O*** | Wilcoxon rank sum | **0.0125** |
| | Transferrin R | Wilcoxon rank sum | 0.31 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor L | Wilcoxon rank sum | 1.00 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.40 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.23 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.82 |
| **Weeks with $\geq 10^3$ *Pasteurella* spp.** | Sex | Wilcoxon rank sum | 0.21 |
| | Vaccine group | Kruskal-Wallis | 0.18 |
| | Transferrin D | Wilcoxon rank sum | 0.46 |
| | Transferrin F2 | Wilcoxon rank sum | 0.41 |
| | Transferrin H1 | Wilcoxon rank sum | 0.38 |
| | Transferrin H2 | Wilcoxon rank sum | 0.97 |
| | **Transferrin O*** | Wilcoxon rank sum | **0.07** |
| | Transferrin R | Wilcoxon rank sum | 0.52 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.13 |
| | Pr. inhibitor L | Wilcoxon rank sum | 1.00 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.81 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.18 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.70 |

* 9 ponies with transferrin O haplotype ranked significantly LOWER scores

Table A2.12 continued

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Weeks with ≥$10^4$ *Pasteurella* spp.** | Sex | Wilcoxon rank sum | 0.08 |
| | Vaccine group | Kruskal-Wallis | 0.16 |
| | Transferrin D | Wilcoxon rank sum | 0.49 |
| | Transferrin F2 | Wilcoxon rank sum | 0.20 |
| | Transferrin H1 | Wilcoxon rank sum | 0.19 |
| | Transferrin H2 | Wilcoxon rank sum | 0.23 |
| | **Transferrin O*** | Wilcoxon rank sum | **0.055** |
| | Transferrin R | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.24 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.97 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.36 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.30 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.72 |
| **Weeks with ≥$10^5$ *Pasteurella* spp.** | Sex | Wilcoxon rank sum | 0.17 |
| | Vaccine group | Kruskal-Wallis | 0.26 |
| | Transferrin D | Wilcoxon rank sum | 0.61 |
| | Transferrin F2 | Wilcoxon rank sum | 0.18 |
| | Transferrin H1 | Wilcoxon rank sum | 0.40 |
| | Transferrin H2 | Wilcoxon rank sum | 0.26 |
| | Transferrin O | Wilcoxon rank sum | 0.14 |
| | Transferrin R | Wilcoxon rank sum | 0.20 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.34 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.61 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.71 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.71 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.75 |
| **$Log_{10}$ *Pasteurella* spp.at week 26** | Sex | Wilcoxon rank sum | 0.37 |
| | Vaccine group | Kruskal-Wallis | 0.36 |
| | Transferrin D | Wilcoxon rank sum | 0.48 |
| | Transferrin F2 | Wilcoxon rank sum | 0.09 |
| | Transferrin H1 | Wilcoxon rank sum | 0.57 |
| | Transferrin H2 | Wilcoxon rank sum | 0.34 |
| | Transferrin O | Wilcoxon rank sum | 0.35 |
| | Transferrin R | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor I | Wilcoxon rank sum | 1.00 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.30 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.95 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.75 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.18 |

* 9 ponies with transferrin O haplotype ranked significantly LOWER scores

**Table A2.13: Summary of non-parametric analyses examining differences in tracheal *B. bronchiseptica* cumulative variables according to pony level explanatory variables**

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **$Log_{10}$ total *B. Bronchiseptica*** | Sex | Wilcoxon rank sum | 0.37 |
| | Vaccine group | Kruskal-Wallis | 0.44 |
| | Transferrin D | Wilcoxon rank sum | 0.36 |
| | Transferrin F2 | Wilcoxon rank sum | 0.91 |
| | Transferrin H1 | Wilcoxon rank sum | 0.39 |
| | Transferrin H2 | Wilcoxon rank sum | 0.66 |
| | Transferrin O | Wilcoxon rank sum | 0.42 |
| | Transferrin R | Wilcoxon rank sum | 0.80 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.31 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.81 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.84 |
| **Maximum $Log_{10}$ *B. bronchiseptica*** | Sex | Wilcoxon rank sum | 0.41 |
| | Vaccine group | Kruskal-Wallis | 0.36 |
| | Transferrin D | Wilcoxon rank sum | 0.50 |
| | Transferrin F2 | Wilcoxon rank sum | 0.73 |
| | Transferrin H1 | Wilcoxon rank sum | 0.50 |
| | Transferrin H2 | Wilcoxon rank sum | 0.66 |
| | Transferrin O | Wilcoxon rank sum | 0.40 |
| | Transferrin R | Wilcoxon rank sum | 0.61 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.35 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.14 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.90 |
| | Pr. inhibitor S | Wilcoxon rank sum | 1.00 |
| **Mean $Log_{10}$ *B. bronchiseptica*** | Sex | Wilcoxon rank sum | 0.39 |
| | Vaccine group | Kruskal-Wallis | 0.87 |
| | Transferrin D | Wilcoxon rank sum | 0.51 |
| | Transferrin F2 | Wilcoxon rank sum | 0.27 |
| | Transferrin H1 | Wilcoxon rank sum | 0.09 |
| | Transferrin H2 | Wilcoxon rank sum | 0.75 |
| | Transferrin O | Wilcoxon rank sum | 0.57 |
| | Transferrin R | Wilcoxon rank sum | 0.45 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.83 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.30 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.19 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.40 |
| **Weeks with $\geq 10^3$ *B. bronchiseptica*** | Sex | Wilcoxon rank sum | 0.25 |
| | Vaccine group | Kruskal-Wallis | 0.97 |
| | Transferrin D | Wilcoxon rank sum | 0.41 |
| | Transferrin F2 | Wilcoxon rank sum | 0.46 |
| | Transferrin H1 | Wilcoxon rank sum | 0.35 |
| | Transferrin H2 | Wilcoxon rank sum | 0.76 |
| | Transferrin O | Wilcoxon rank sum | 0.59 |
| | Transferrin R | Wilcoxon rank sum | 0.78 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.91 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.82 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.08 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.07 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.83 |

Table A2.13 continued

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Weeks with ≥$10^4$ *B. bronchiseptica*** | Sex | Wilcoxon rank sum | 0.66 |
| | Vaccine group | Kruskal-Wallis | 0.64 |
| | Transferrin D | Wilcoxon rank sum | 0.25 |
| | Transferrin F2 | Wilcoxon rank sum | 0.60 |
| | Transferrin H1 | Wilcoxon rank sum | 0.17 |
| | Transferrin H2 | Wilcoxon rank sum | 0.84 |
| | Transferrin O | Wilcoxon rank sum | 0.96 |
| | Transferrin R | Wilcoxon rank sum | 0.62 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.63 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.57 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.13 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.08 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.24 |
| **Weeks with ≥$10^5$ *B. bronchiseptica*** | Sex | Wilcoxon rank sum | 0.40 |
| | Vaccine group | Kruskal-Wallis | 0.20 |
| | Transferrin D | Wilcoxon rank sum | 0.76 |
| | Transferrin F2 | Wilcoxon rank sum | 0.73 |
| | Transferrin H1 | Wilcoxon rank sum | 0.29 |
| | Transferrin H2 | Wilcoxon rank sum | 0.64 |
| | Transferrin O | Wilcoxon rank sum | 0.98 |
| | Transferrin R | Wilcoxon rank sum | 0.89 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.89 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.29 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.17 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.09 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.09 |
| **$Log_{10}$ *B. bronchiseptica* at week 26** | Sex | Wilcoxon rank sum | 0.92 |
| | Vaccine group | Kruskal-Wallis | 0.75 |
| | Transferrin D | Wilcoxon rank sum | 0.68 |
| | Transferrin F2 | Wilcoxon rank sum | 0.16 |
| | Transferrin H1 | Wilcoxon rank sum | 0.62 |
| | Transferrin H2 | Wilcoxon rank sum | 0.11 |
| | Transferrin O | Wilcoxon rank sum | 0.03 |
| | Transferrin R | Wilcoxon rank sum | 0.56 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.84 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.62 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.79 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.79 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.51 |

**Table A2.14: Summary of non-parametric analyses examining differences in tracheal non-haemolytic *Streptococcus* spp. cumulative variables for pony level variables**

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **$Log_{10}$ total non-haemolytic *Strep.* spp.** | Sex | Wilcoxon rank sum | 0.86 |
| | Vaccine group | Kruskal-Wallis | 0.55 |
| | Transferrin D | Wilcoxon rank sum | 0.18 |
| | Transferrin F2 | Wilcoxon rank sum | 0.16 |
| | Transferrin H1 | Wilcoxon rank sum | 0.06 |
| | Transferrin H2 | Wilcoxon rank sum | 0.85 |
| | Transferrin O | Wilcoxon rank sum | 0.11 |
| | Transferrin R | Wilcoxon rank sum | 0.80 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.51 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.47 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.34 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.07 |
| **Maximum $Log_{10}$ non-haemolytic *Strep.* spp.** | Sex | Wilcoxon rank sum | 0.71 |
| | Vaccine group | Kruskal-Wallis | 0.47 |
| | Transferrin D | Wilcoxon rank sum | 0.15 |
| | Transferrin F2 | Wilcoxon rank sum | 0.40 |
| | Transferrin H1 | Wilcoxon rank sum | 0.07 |
| | Transferrin H2 | Wilcoxon rank sum | 0.59 |
| | Transferrin O | Wilcoxon rank sum | 0.08 |
| | Transferrin R | Wilcoxon rank sum | 0.90 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.66 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.52 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.47 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.08 |
| **Mean $Log_{10}$ non-haemolytic *Strep.* spp.** | Sex | Wilcoxon rank sum | 0.16 |
| | **Vaccine group** | **Kruskal-Wallis** | **0.0345** |
| | Transferrin D | Wilcoxon rank sum | 0.39 |
| | **Transferrin F2*** | **Wilcoxon rank sum** | **0.0006** |
| | Transferrin H1 | Wilcoxon rank sum | 0.57 |
| | Transferrin H2 | Wilcoxon rank sum | 0.28 |
| | **Transferrin O**** | **Wilcoxon rank sum** | **0.0301** |
| | Transferrin R | Wilcoxon rank sum | 0.66 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.36 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.89 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.15 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.23 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.30 |
| **Weeks with $\geq 10^3$ non-haemolytic *Strep.* spp.** | Sex | Wilcoxon rank sum | 0.10 |
| | **Vaccine group** | **Kruskal-Wallis** | **0.0245** |
| | Transferrin D | Wilcoxon rank sum | 0.40 |
| | **Transferrin F2*** | **Wilcoxon rank sum** | **0.0009** |
| | Transferrin H1 | Wilcoxon rank sum | 0.77 |
| | Transferrin H2 | Wilcoxon rank sum | 0.32 |
| | **Transferrin O**** | **Wilcoxon rank sum** | **0.0401** |
| | Transferrin R | Wilcoxon rank sum | 0.59 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.21 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.83 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.12 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.40 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.31 |

* 14 ponies with transferrin F2 haplotype ranked significantly LOWER scores

** 9 ponies with transferrin O haplotype ranked significantly HIGHER scores

Table A2.14 continued

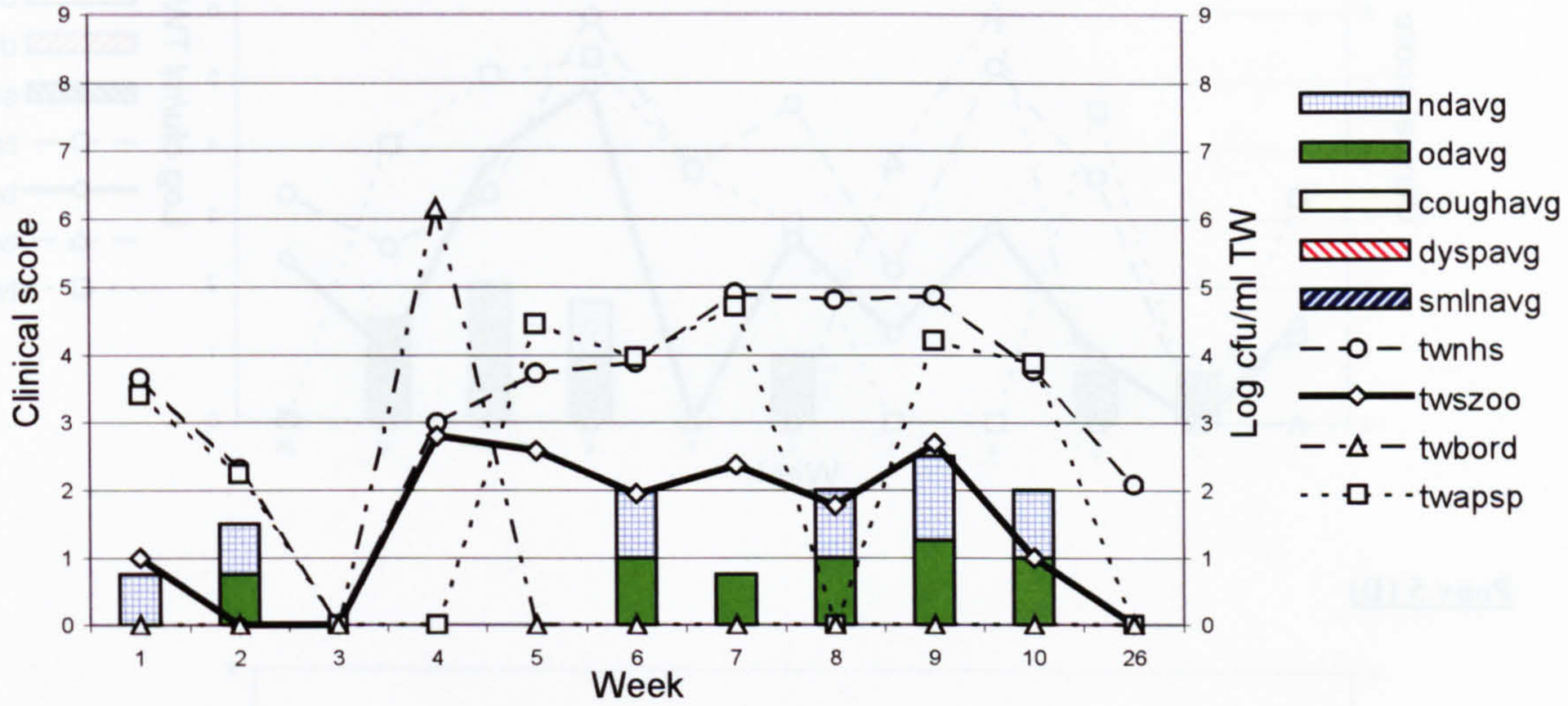
| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Weeks with ≥$10^4$ non-haemolytic *Strep.* spp.** | Sex | Wilcoxon rank sum | 0.44 |
| | Vaccine group | Kruskal-Wallis | 0.07 |
| | Transferrin D | Wilcoxon rank sum | 0.98 |
| | **Transferrin F2*** | **Wilcoxon rank sum** | **0.0083** |
| | Transferrin H1 | Wilcoxon rank sum | 0.31 |
| | Transferrin H2 | Wilcoxon rank sum | 0.36 |
| | **Transferrin O**** | **Wilcoxon rank sum** | **0.0435** |
| | Transferrin R | Wilcoxon rank sum | 0.61 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.10 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.89 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.19 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.31 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.21 |
| **Weeks with ≥$10^5$ non-haemolytic *Strep.* spp.** | Sex | Wilcoxon rank sum | 0.91 |
| | Vaccine group | Kruskal-Wallis | 0.18 |
| | Transferrin D | Wilcoxon rank sum | 0.29 |
| | Transferrin F2 | Wilcoxon rank sum | 0.29 |
| | Transferrin H1 | Wilcoxon rank sum | 0.13 |
| | Transferrin H2 | Wilcoxon rank sum | 0.37 |
| | Transferrin O | Wilcoxon rank sum | 0.07 |
| | Transferrin R | Wilcoxon rank sum | 0.56 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.46 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.36 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.54 |
| | Pr. inhibitor R | Wilcoxon rank sum | 0.54 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.23 |
| **$Log_{10}$ non-haemolytic *Strep.* spp. at week 26** | Sex | Wilcoxon rank sum | 0.46 |
| | Vaccine group | Kruskal-Wallis | 0.21 |
| | Transferrin D | Wilcoxon rank sum | 0.95 |
| | Transferrin F2 | Wilcoxon rank sum | 0.11 |
| | Transferrin H1 | Wilcoxon rank sum | 0.49 |
| | Transferrin H2 | Wilcoxon rank sum | 0.67 |
| | Transferrin O | Wilcoxon rank sum | 0.68 |
| | Transferrin R | Wilcoxon rank sum | 0.21 |
| | Pr. inhibitor I | Wilcoxon rank sum | 0.56 |
| | Pr. inhibitor L | Wilcoxon rank sum | 0.40 |
| | Pr. inhibitor L2 | Wilcoxon rank sum | 0.25 |
| | Pr. inhibitor R | Wilcoxon rank sum | 1.00 |
| | Pr. inhibitor S | Wilcoxon rank sum | 0.35 |

* 14 ponies with transferrin F2 haplotype ranked significantly LOWER scores

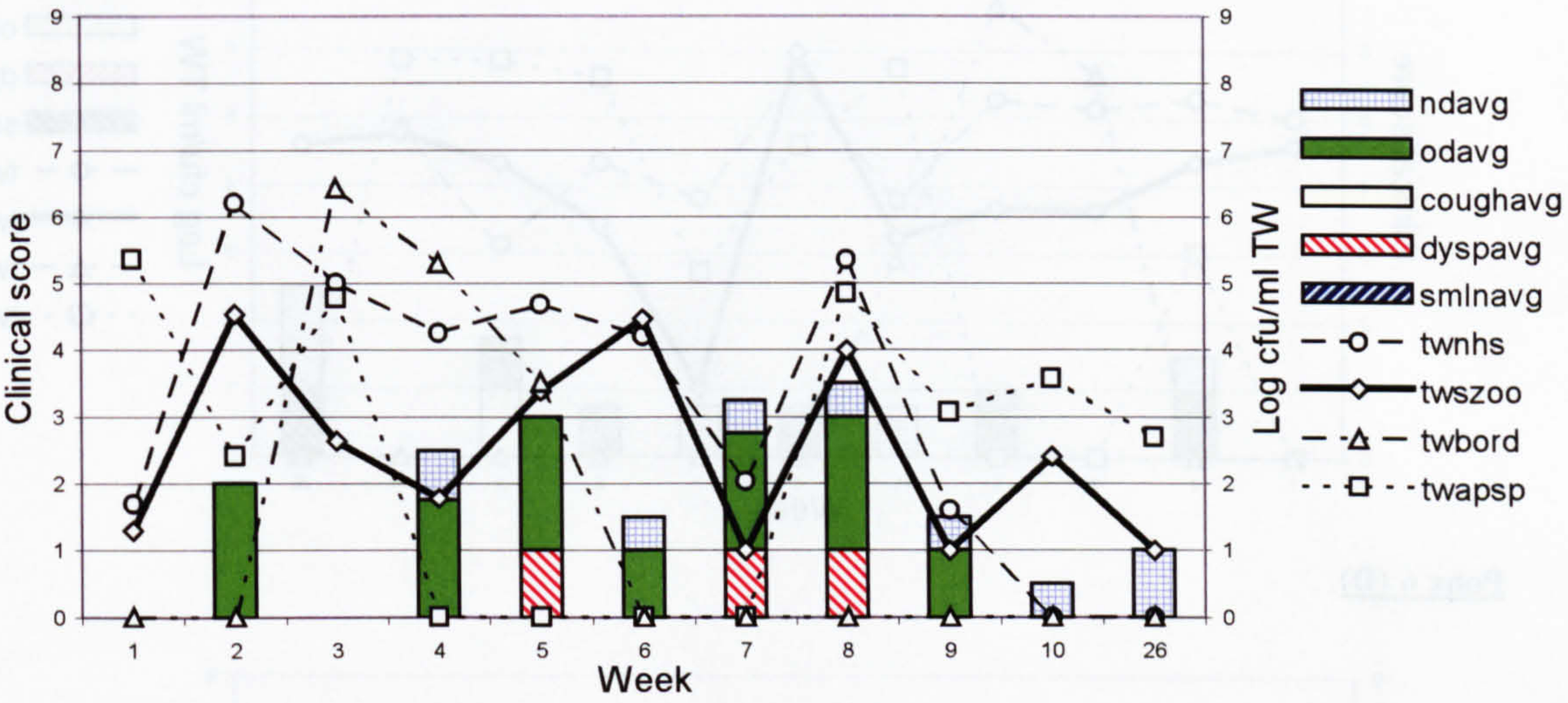
** 9 ponies with transferrin O haplotype ranked significantly HIGHER scores

# Figure A2.6: Summary of weekly clinical signs and TW bacterial counts by pony

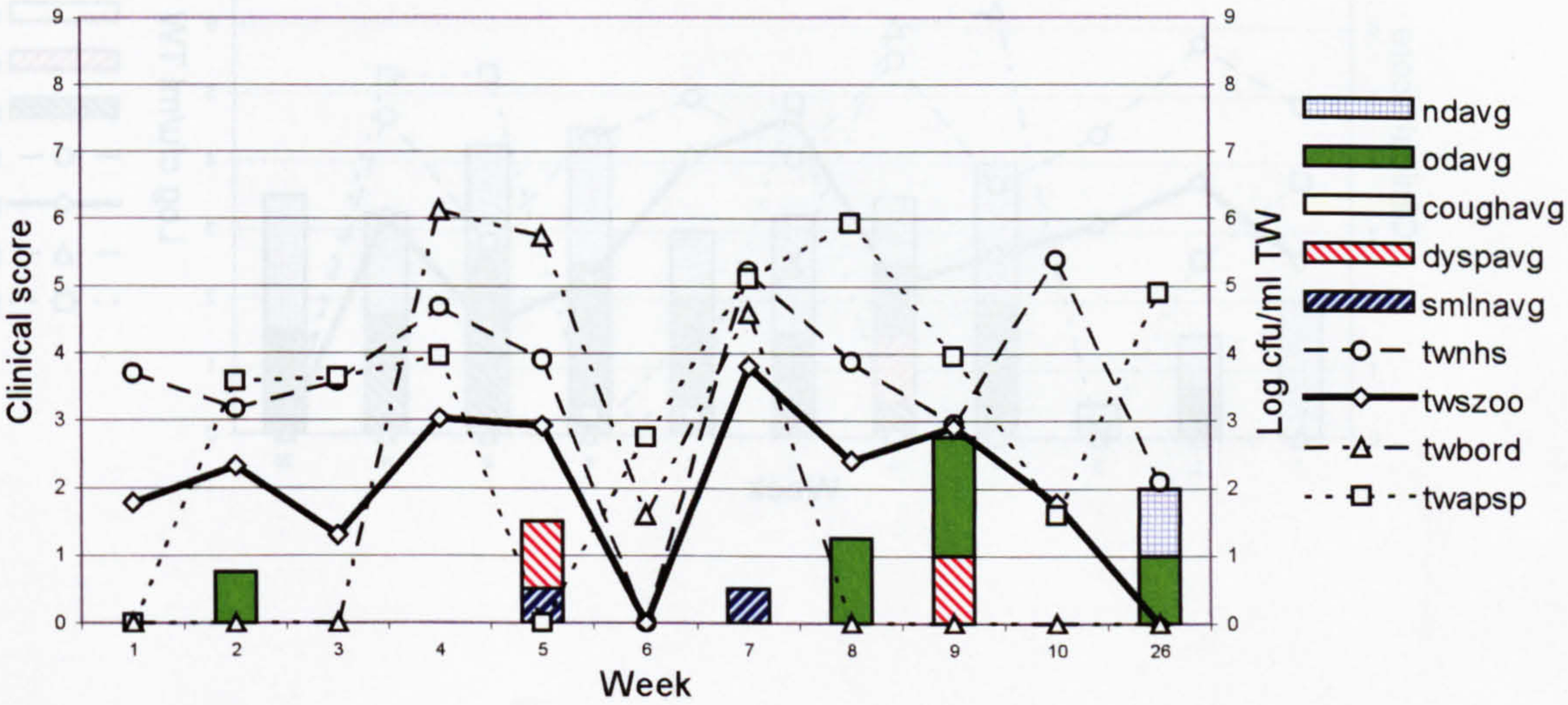
## Pony 1 (transferrin D phenotype)



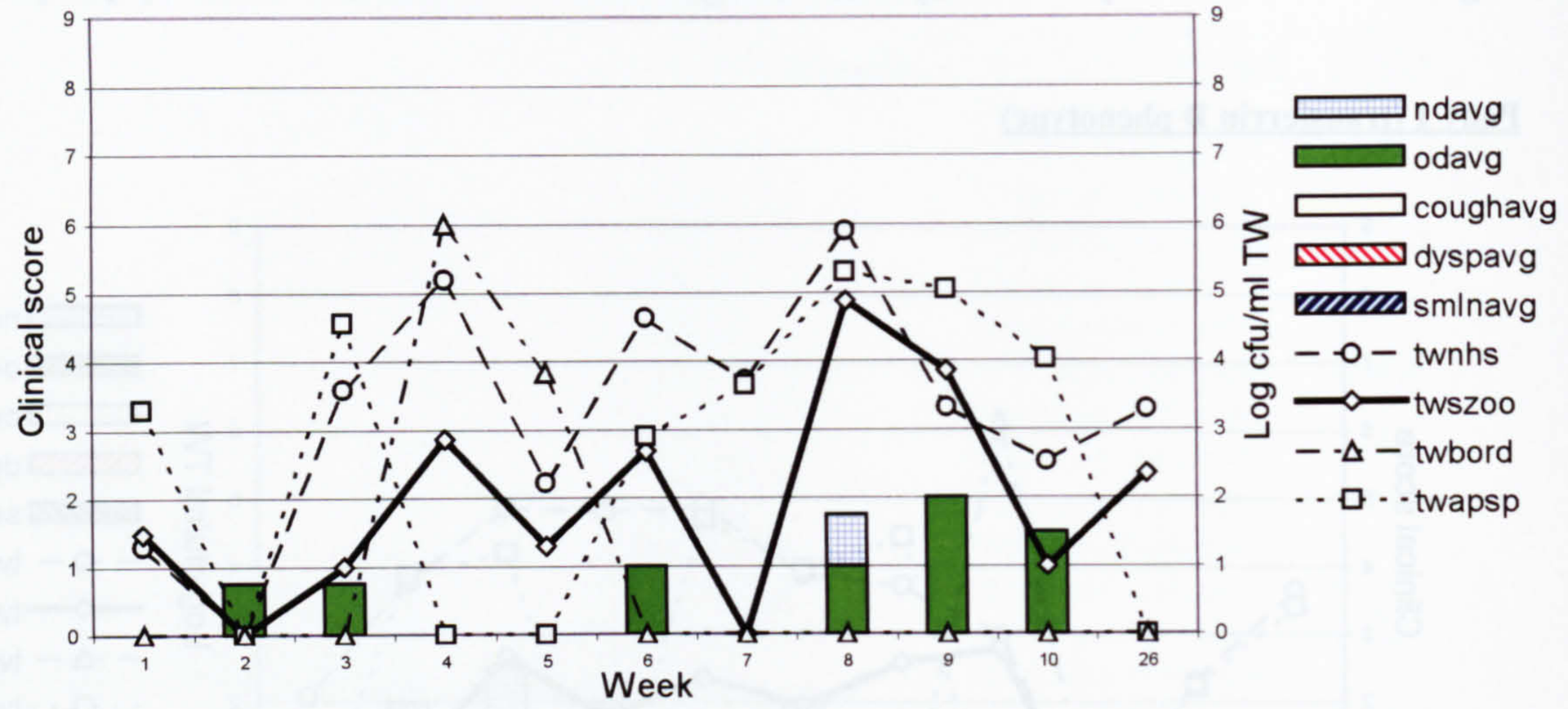

## Pony 2 (D)



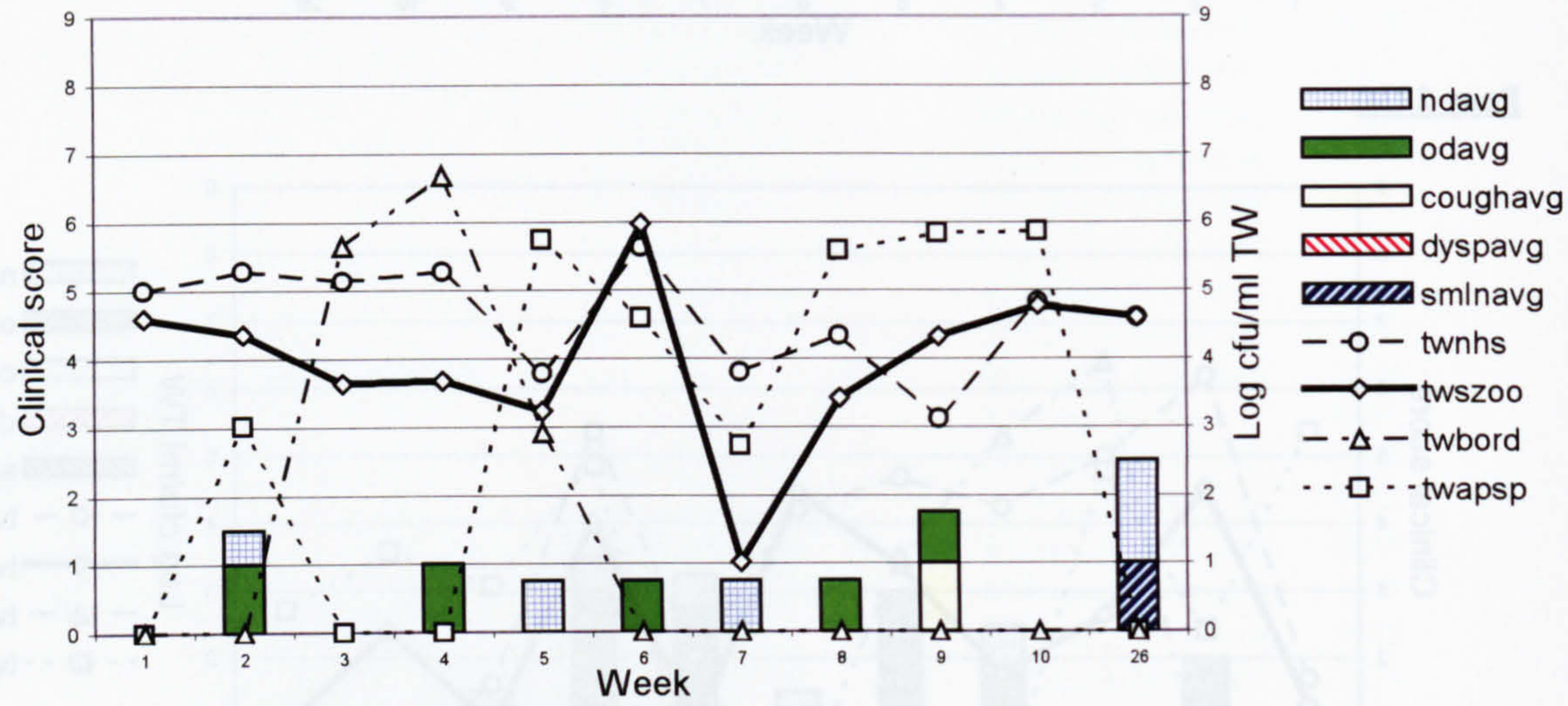

## Pony 3 (F2)



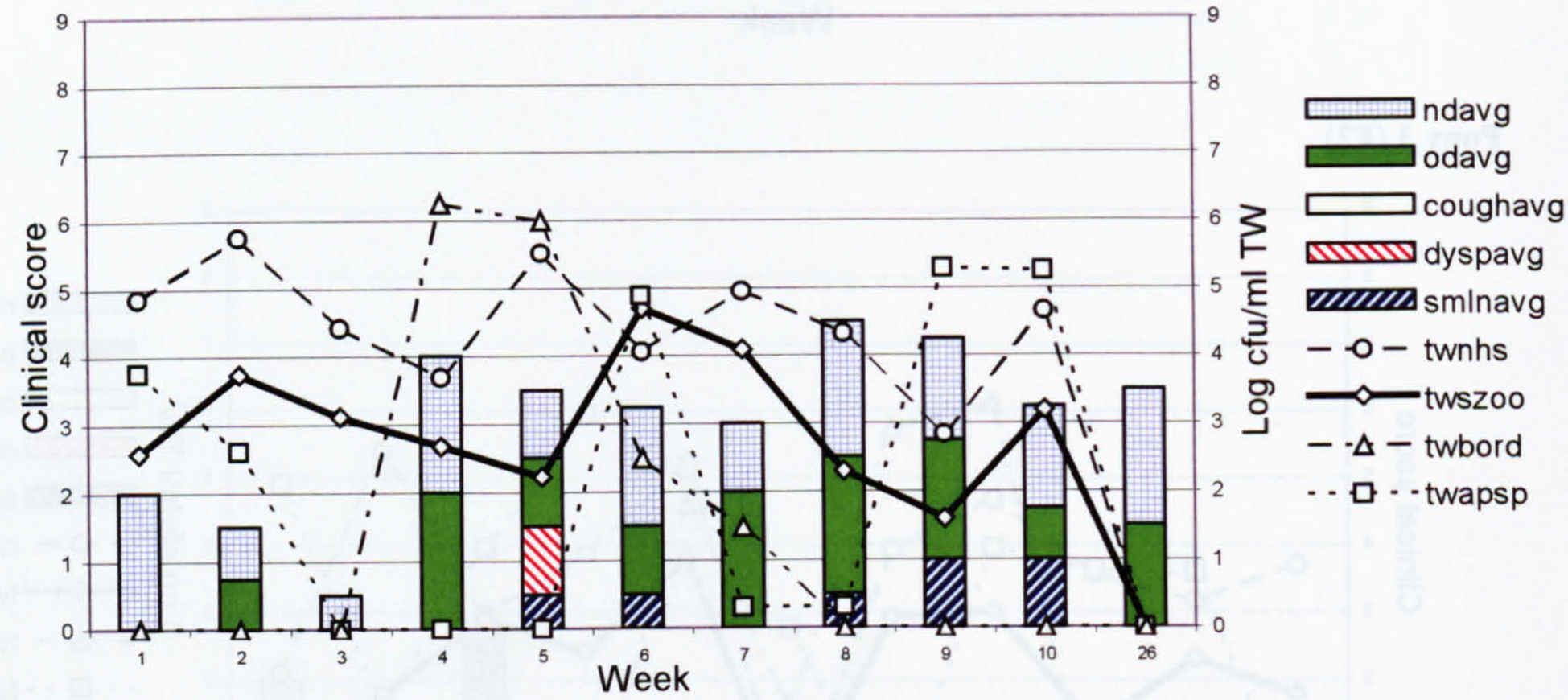
**Pony 4 (D)**



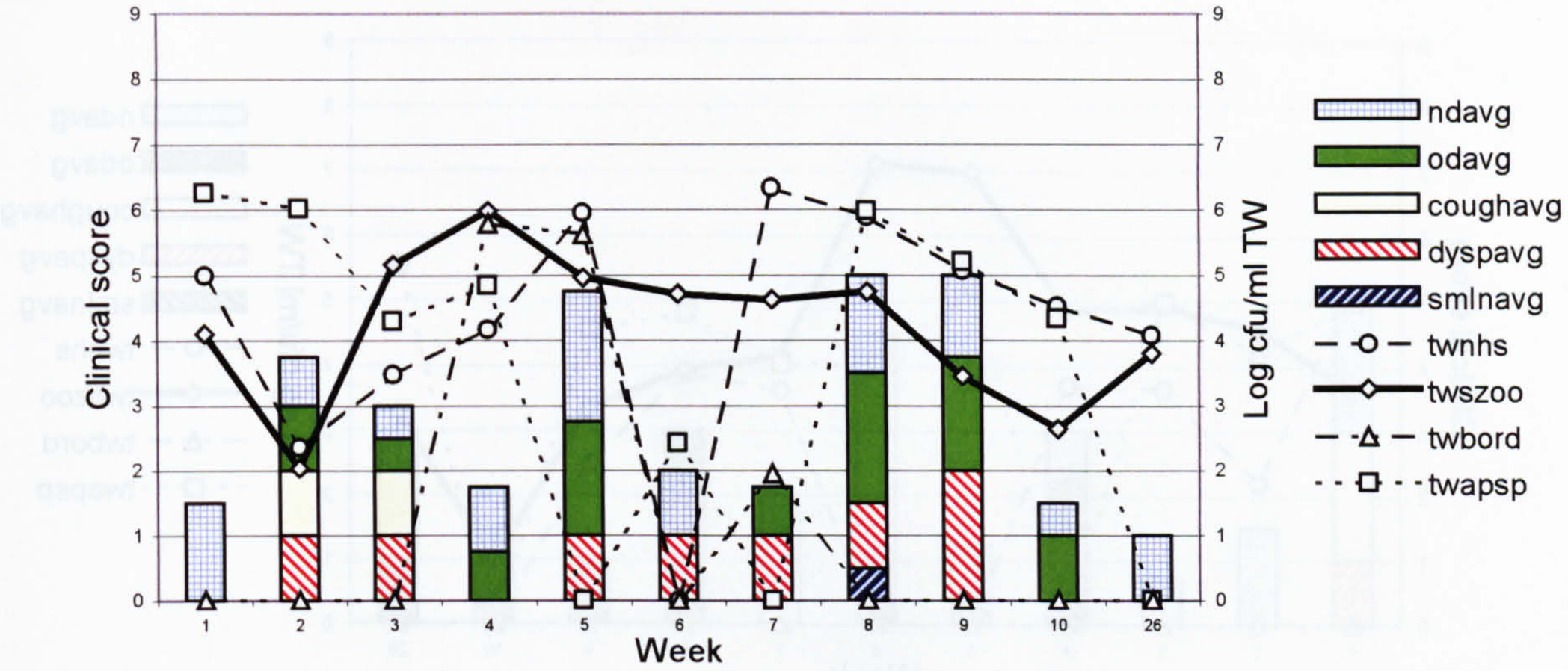

**Pony 5 (D)**



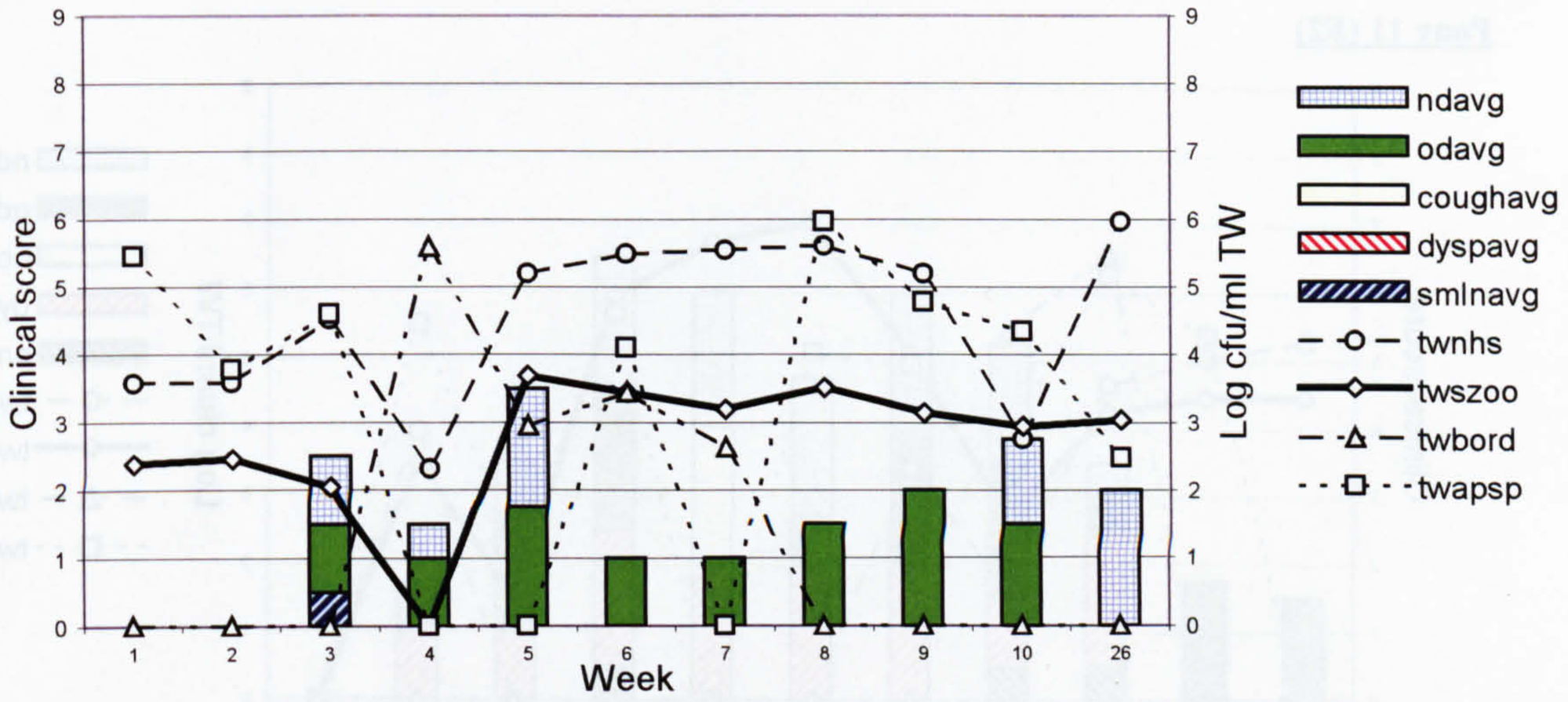

**Pony 6 (D)**



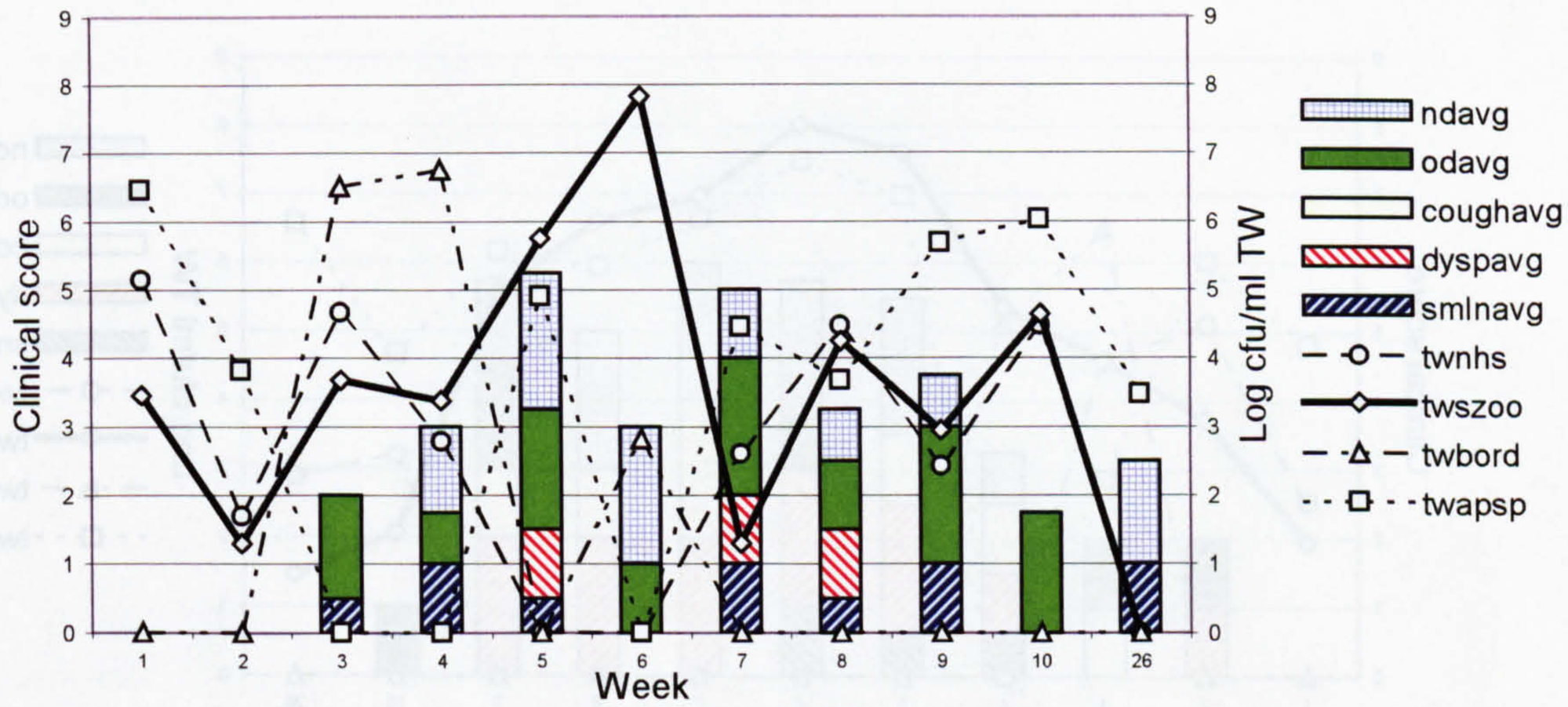
**Pony 7 (D)**



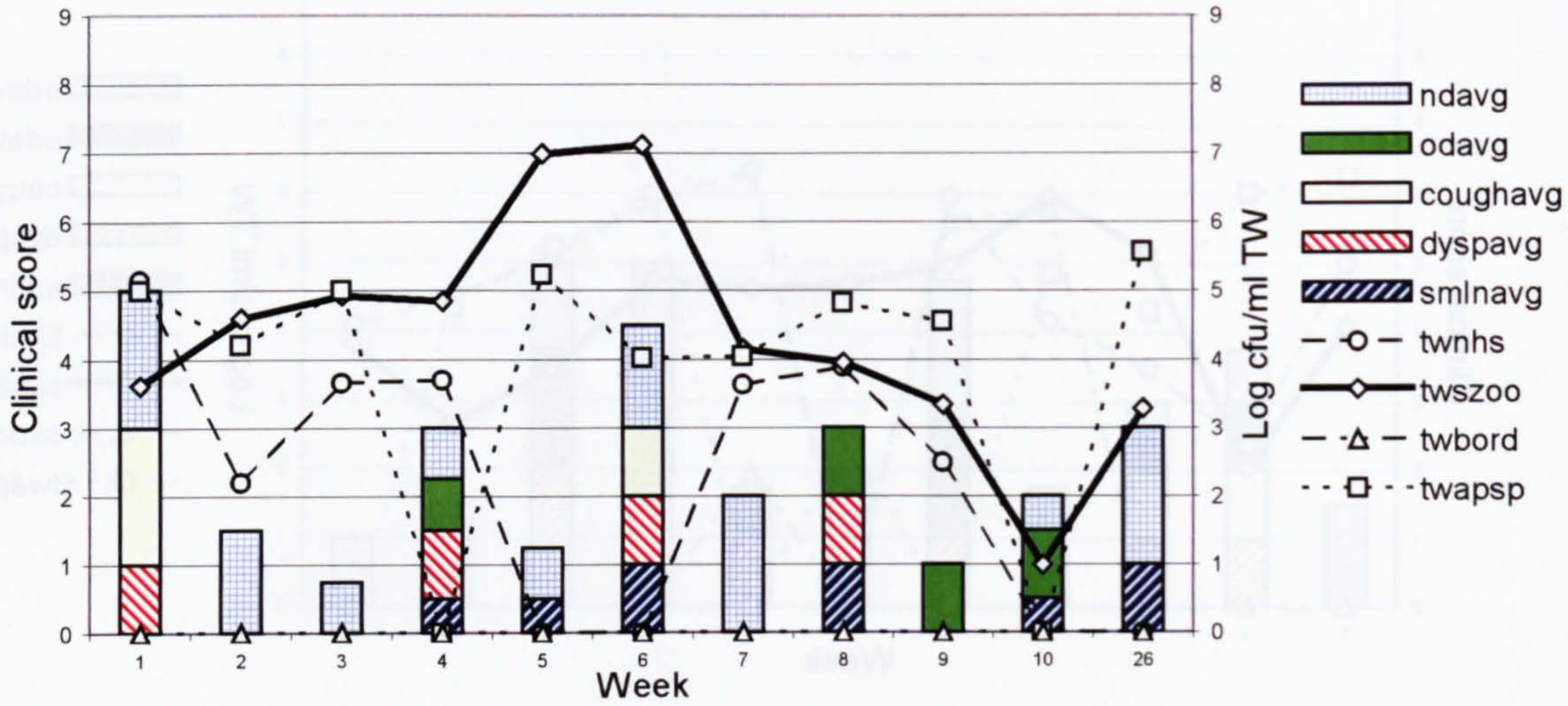

**Pony 8 (F2)**



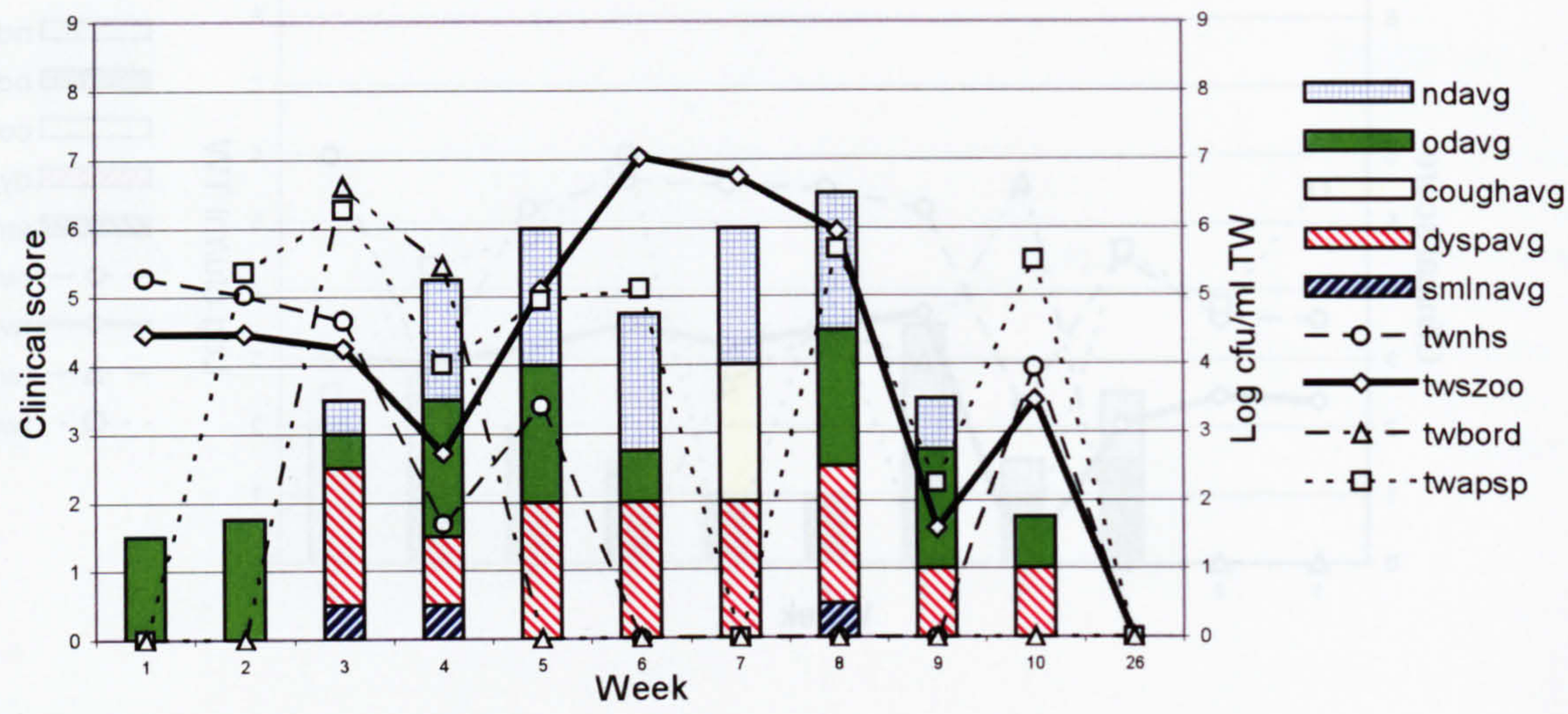

**Pony 9 (D)**



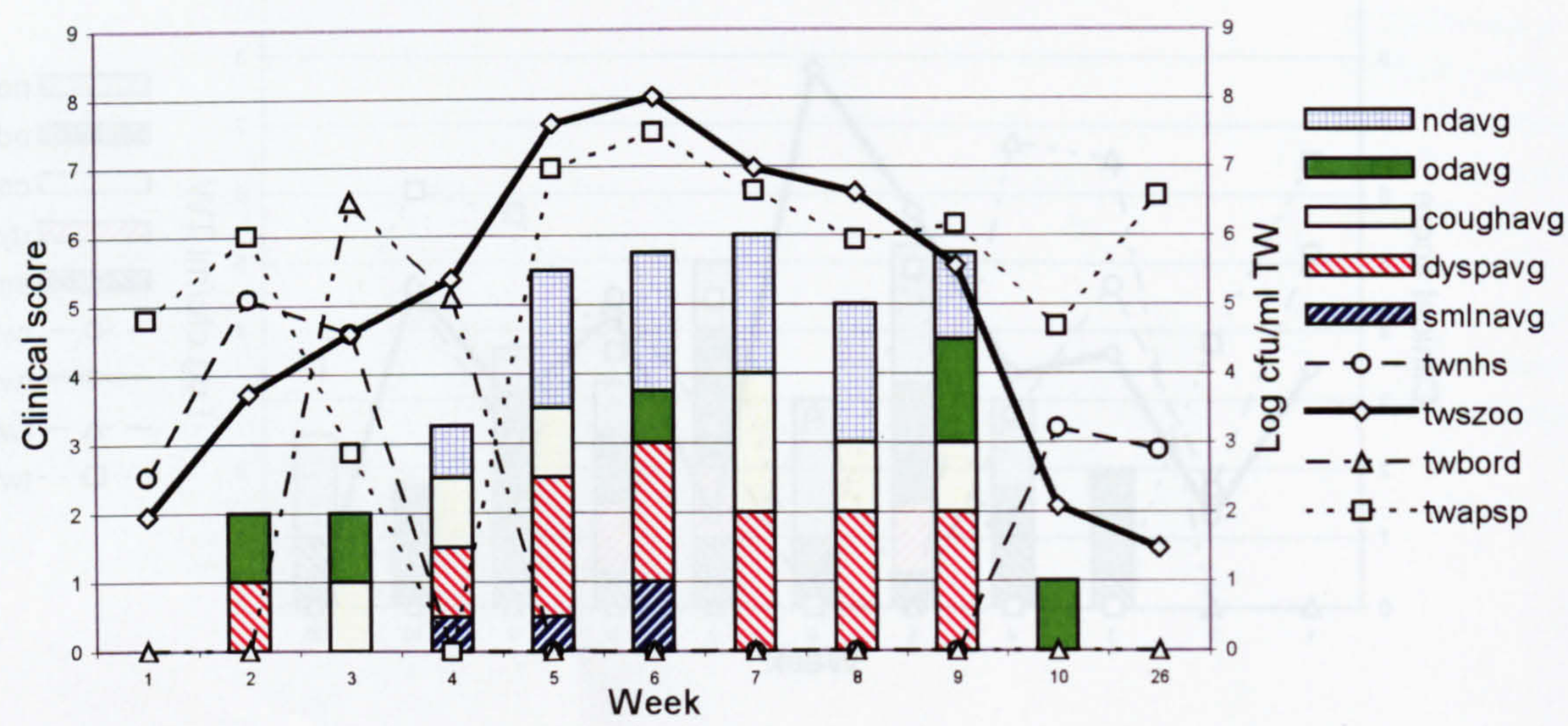
**Pony 10 (DF2)**



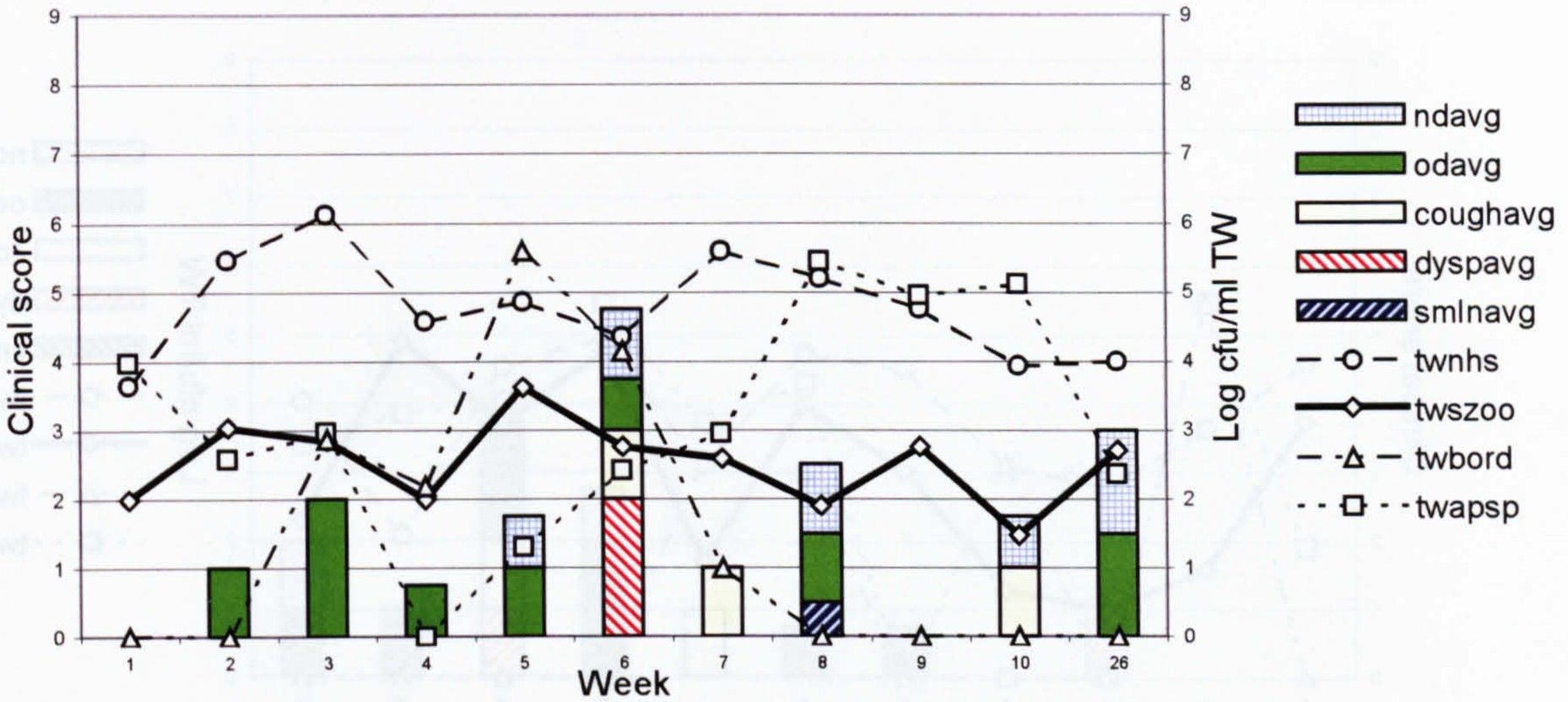

**Pony 11 (F2)**



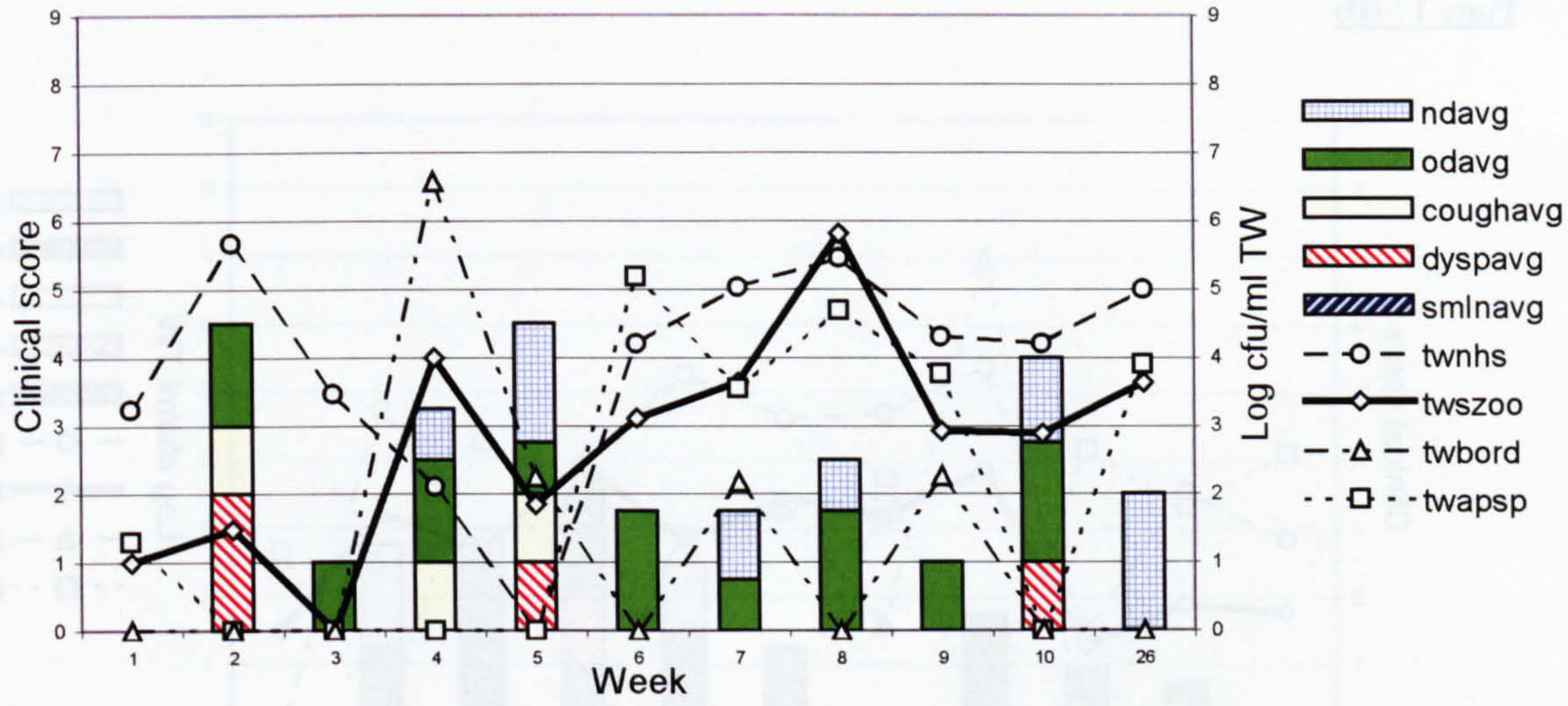

**Pony 12 (DF2)**



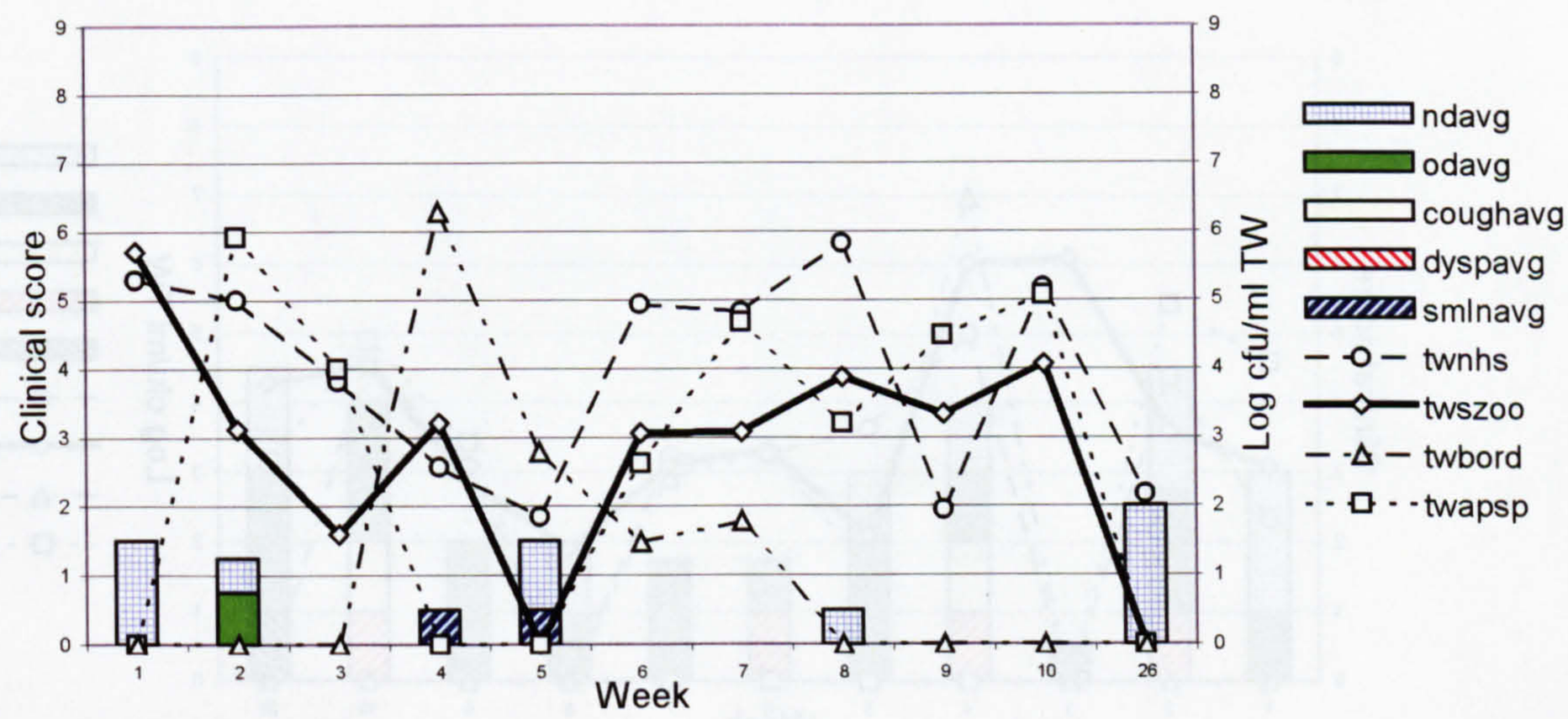
**Pony 13 (D)**



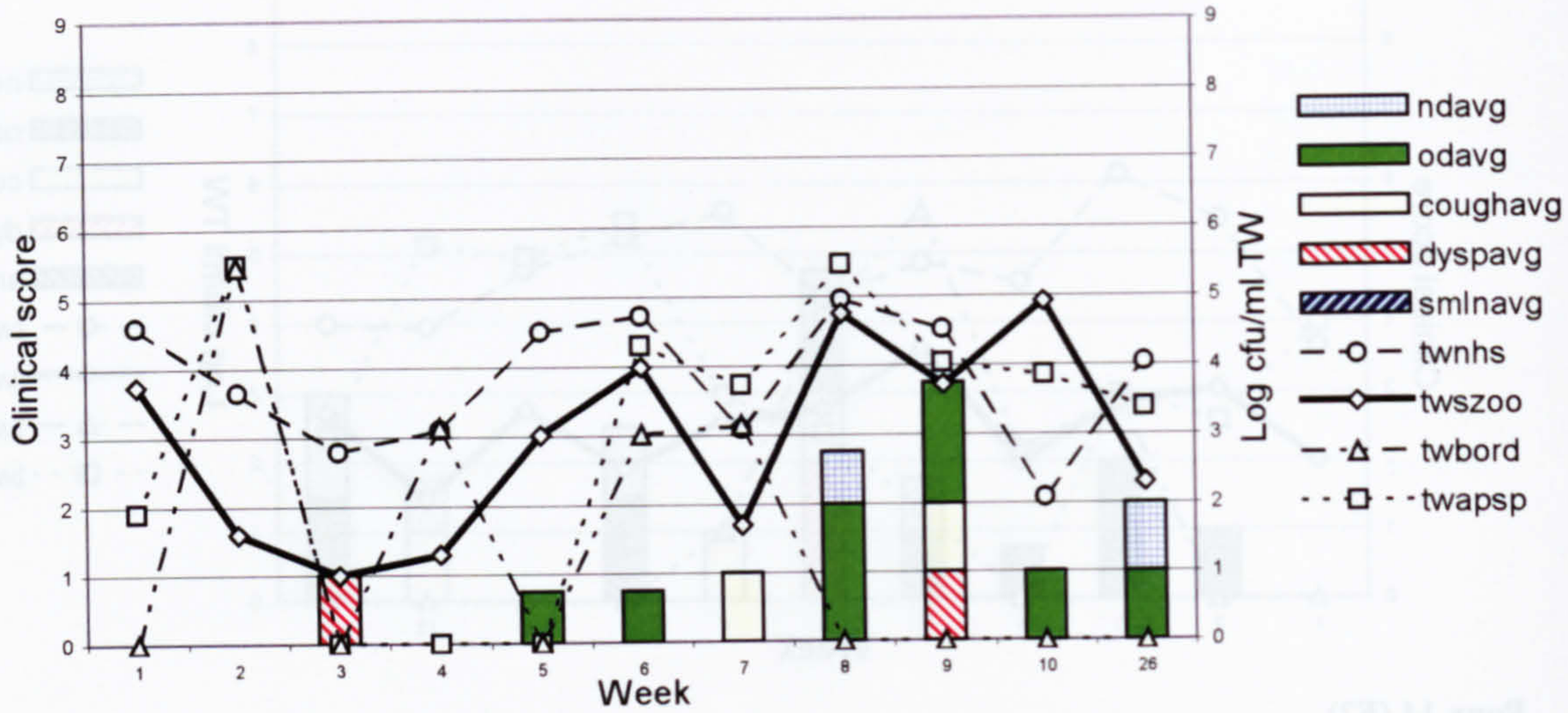

**Pony 14 (F2)**



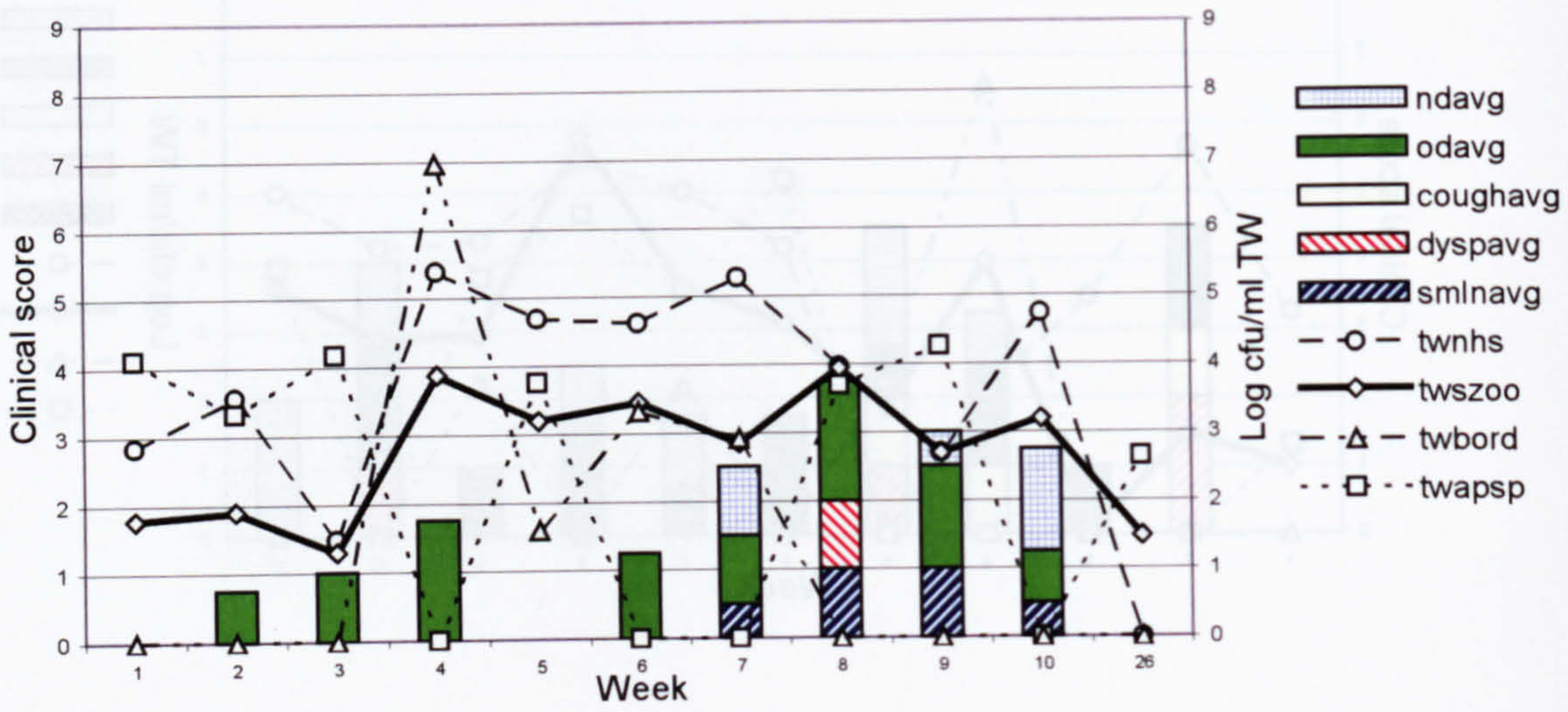

**Pony 15 (D)**



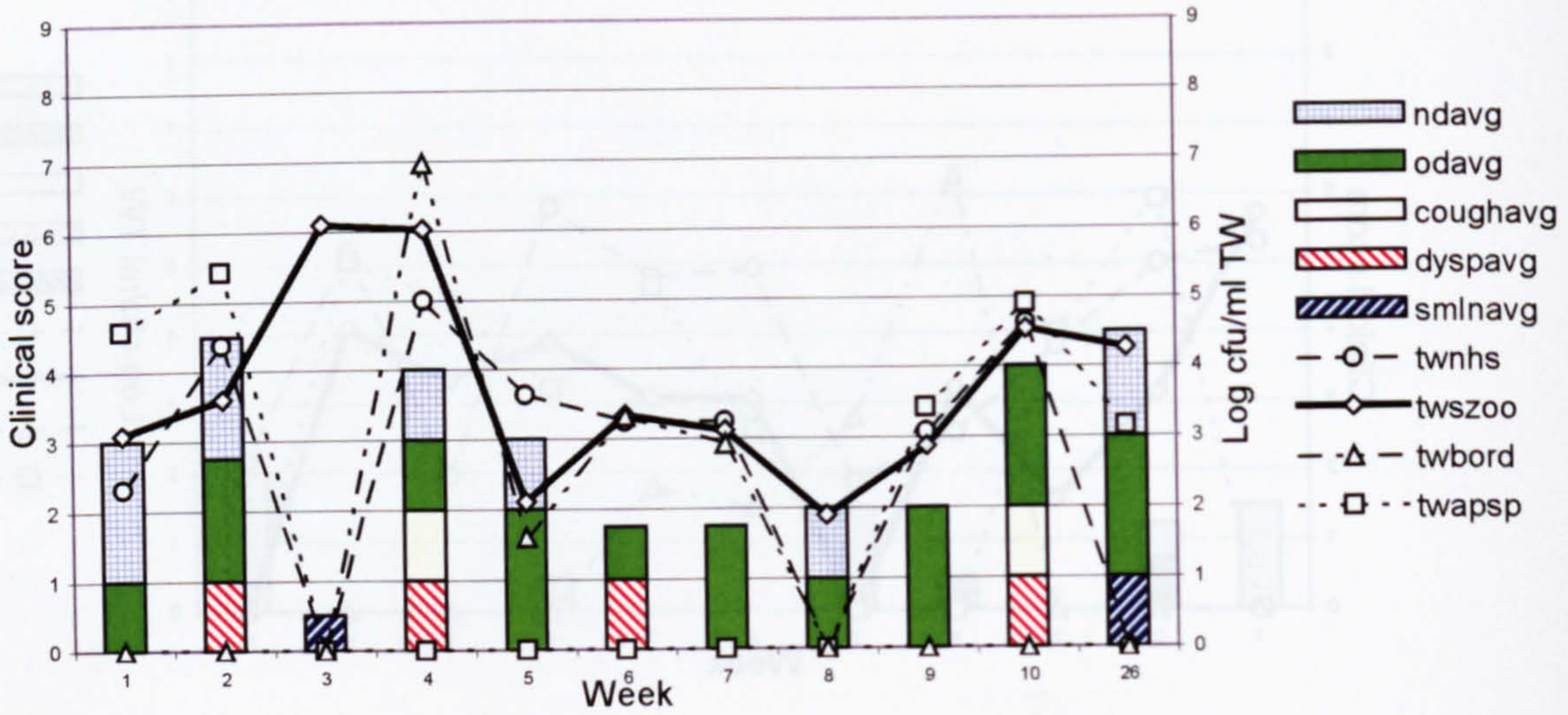
**Pony 16 (D)**



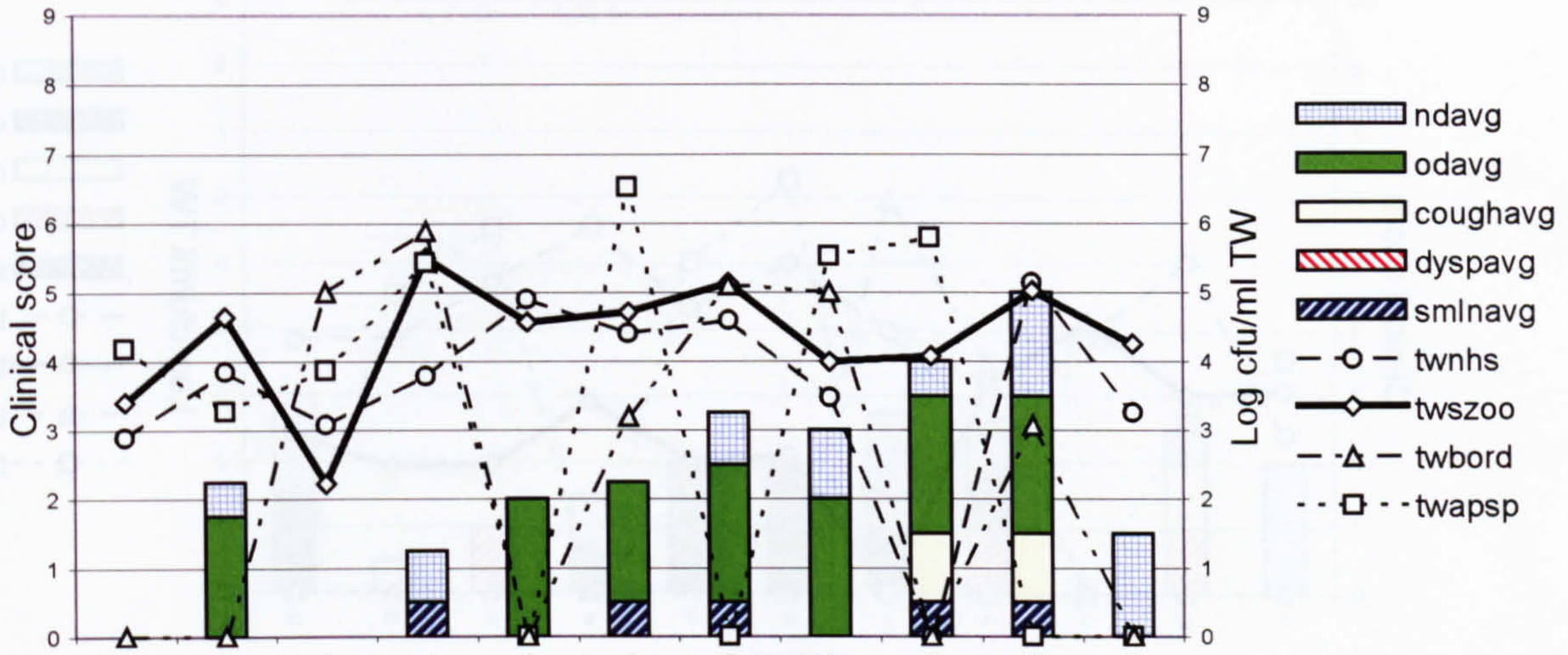

**Pony 17 (D)**



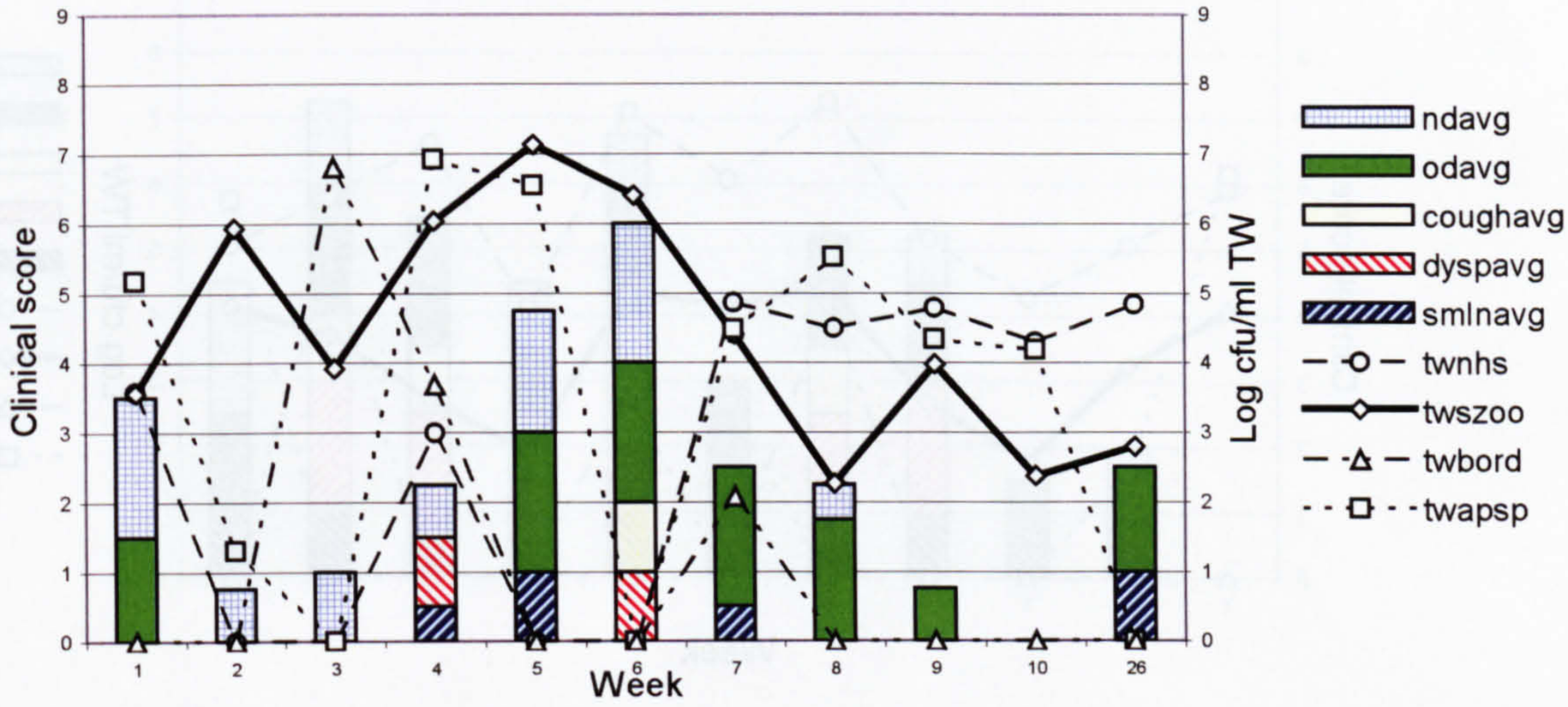

**Pony 18 (F2)**



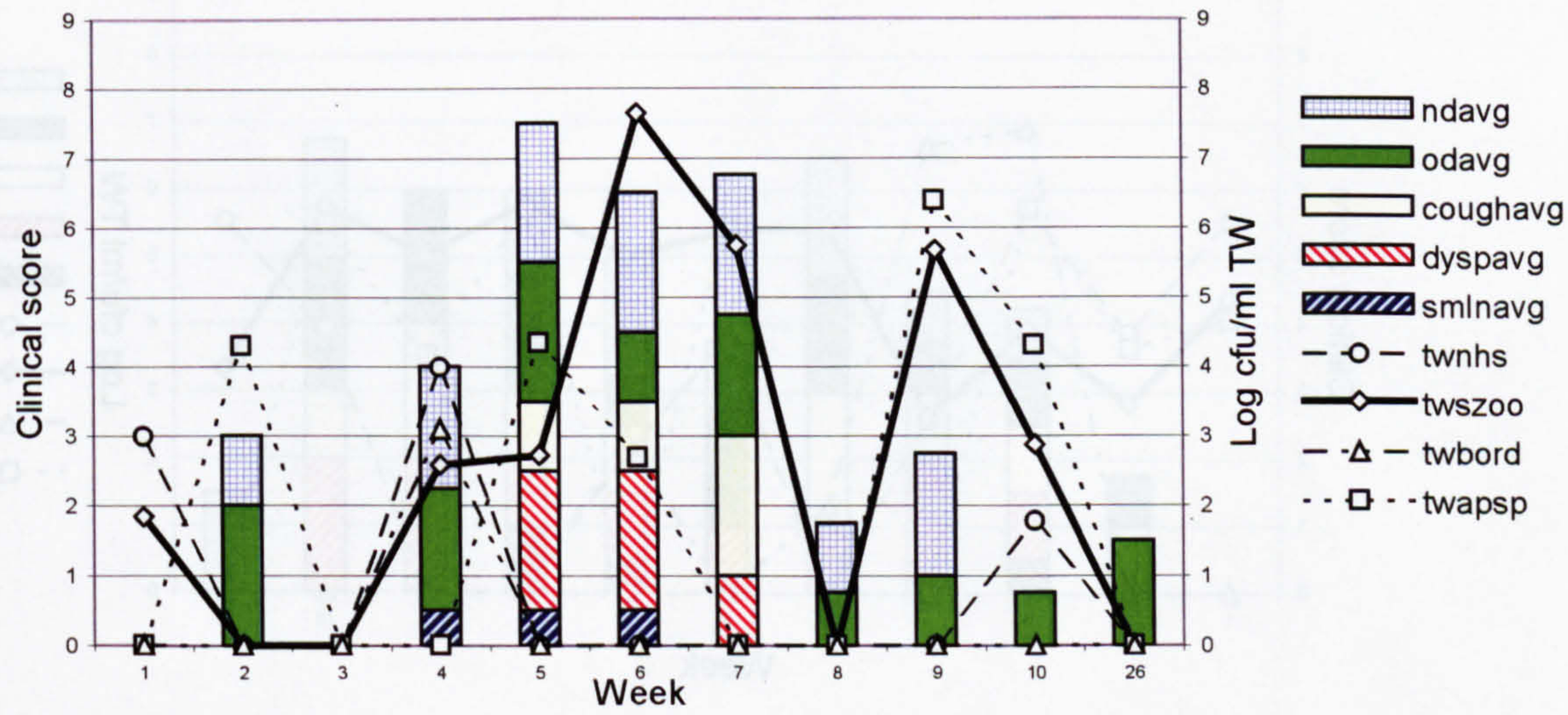
**Pony 19 (D)**



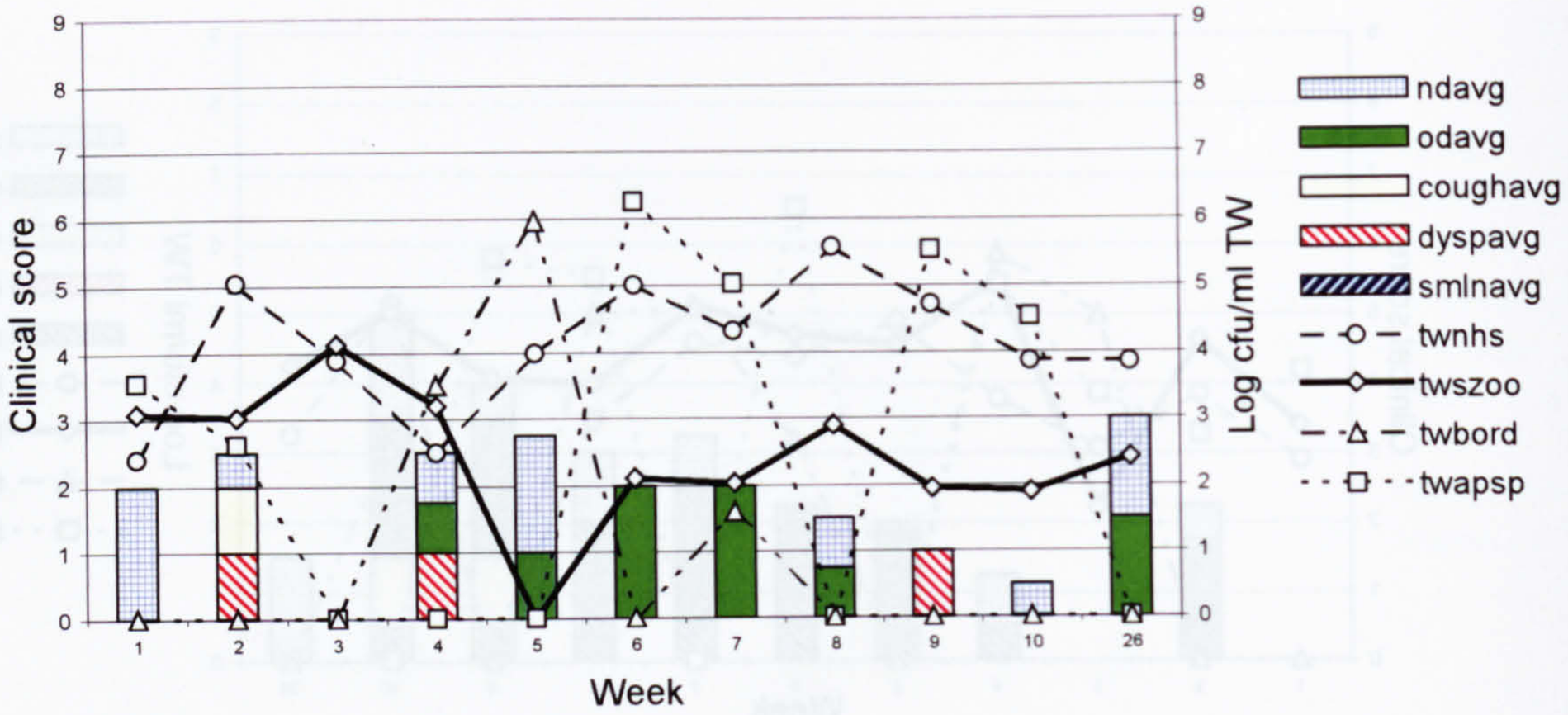

**Pony 20 (D)**



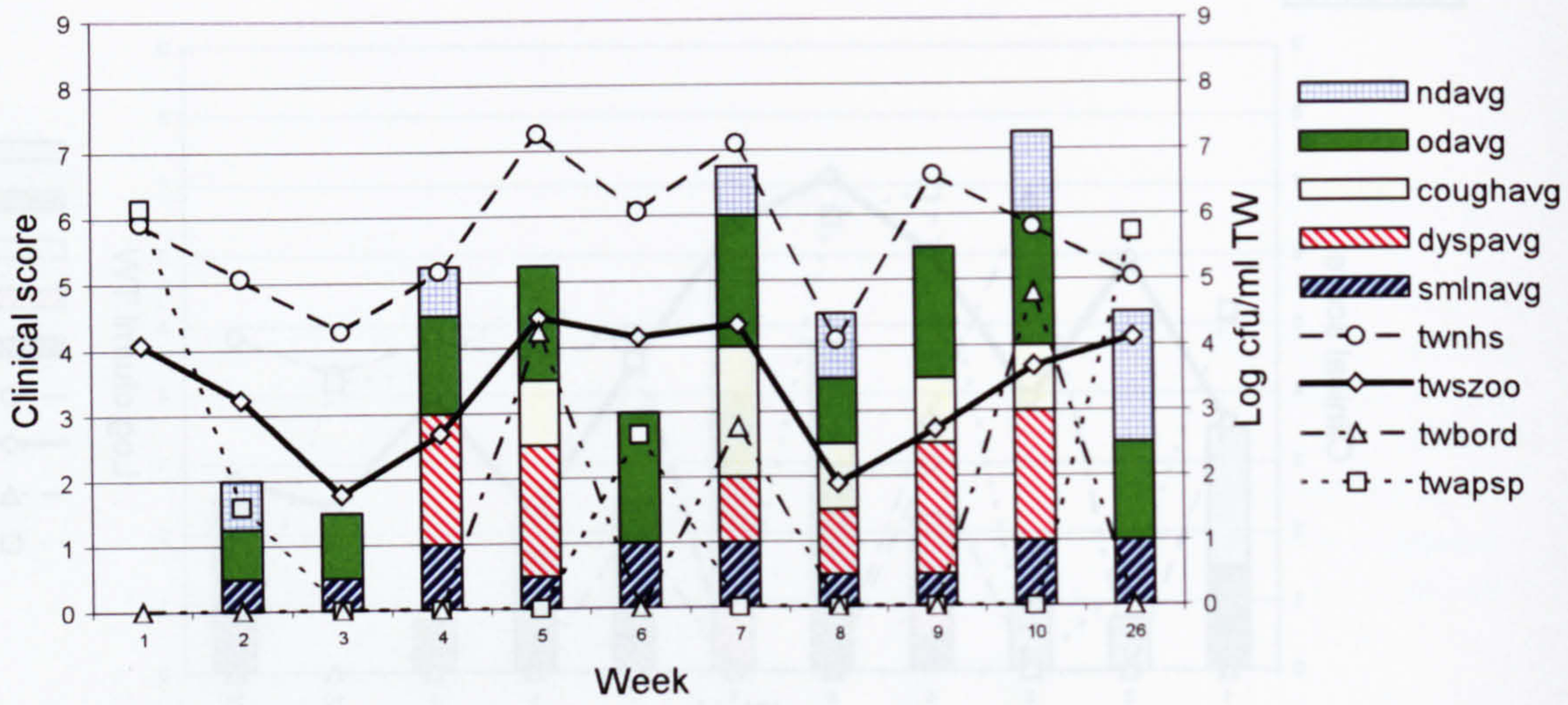

**Pony 21 (DF2)**



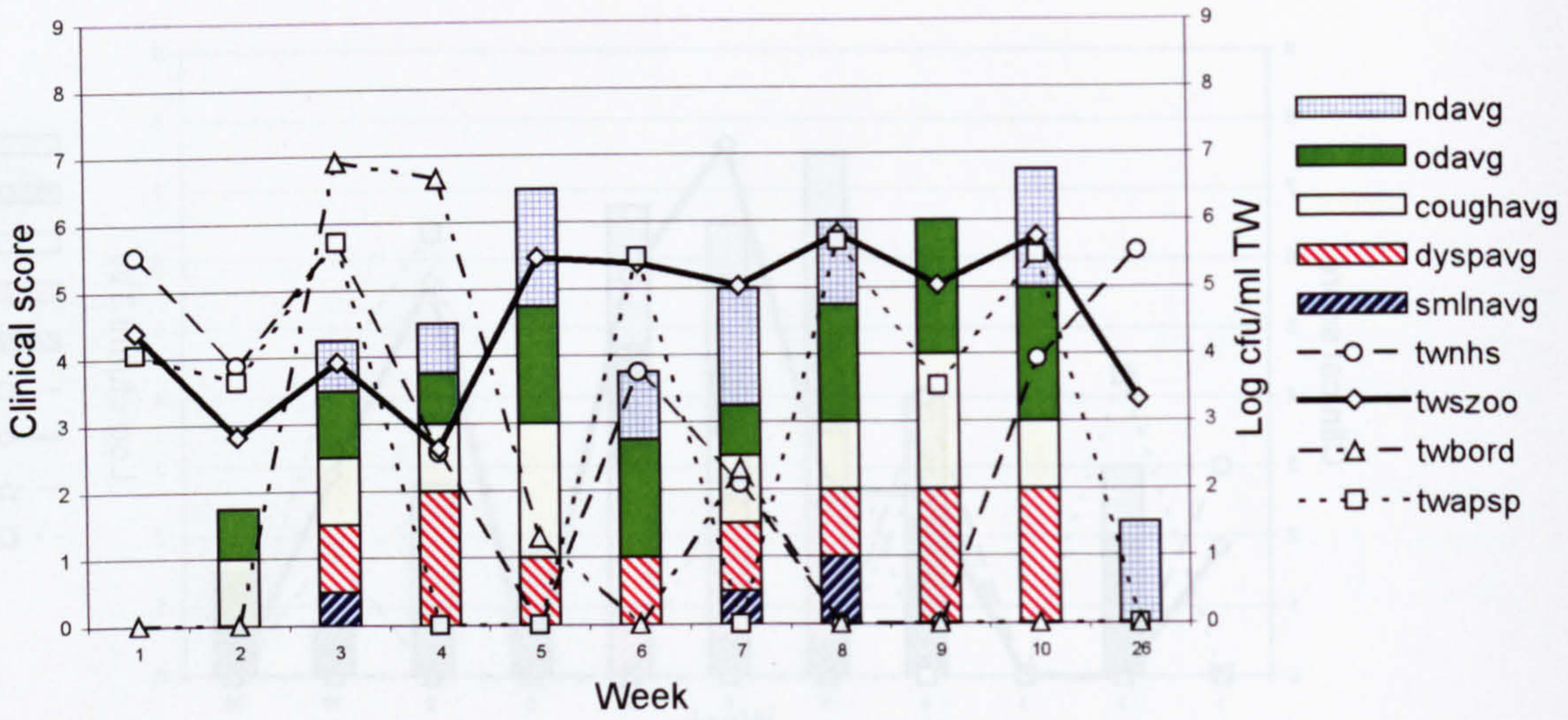
**Pony 22 (D)**



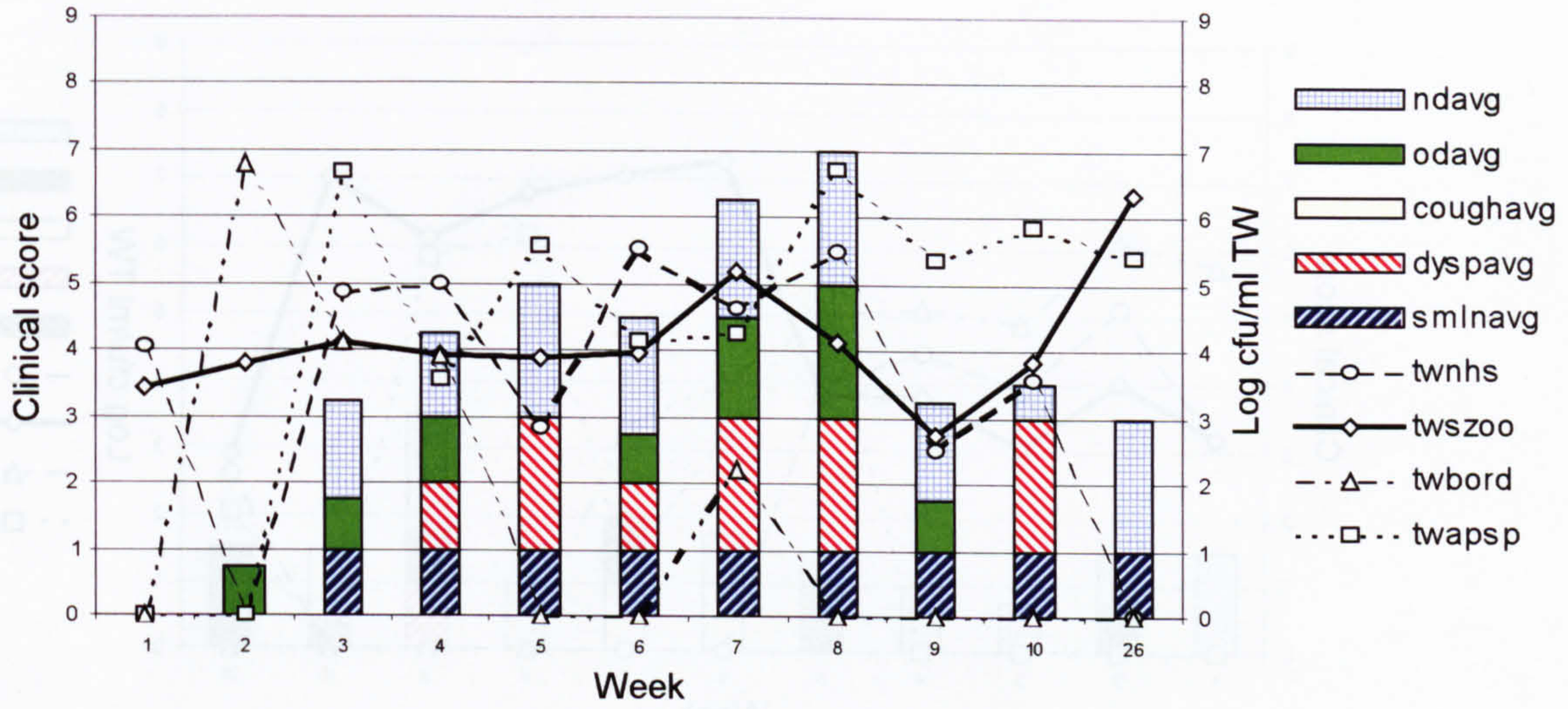

**Pony 23 (OH2)**



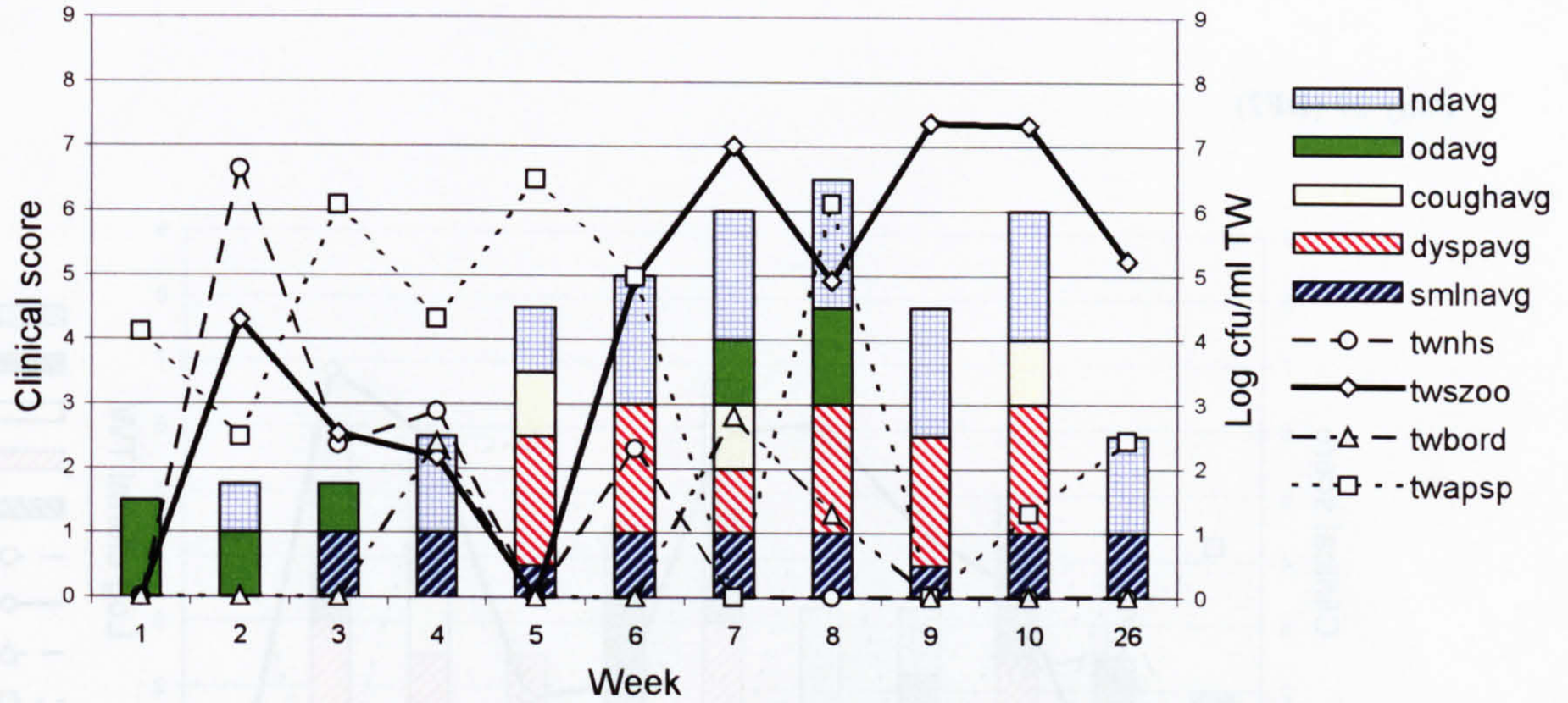

**Pony 24 (F2)**



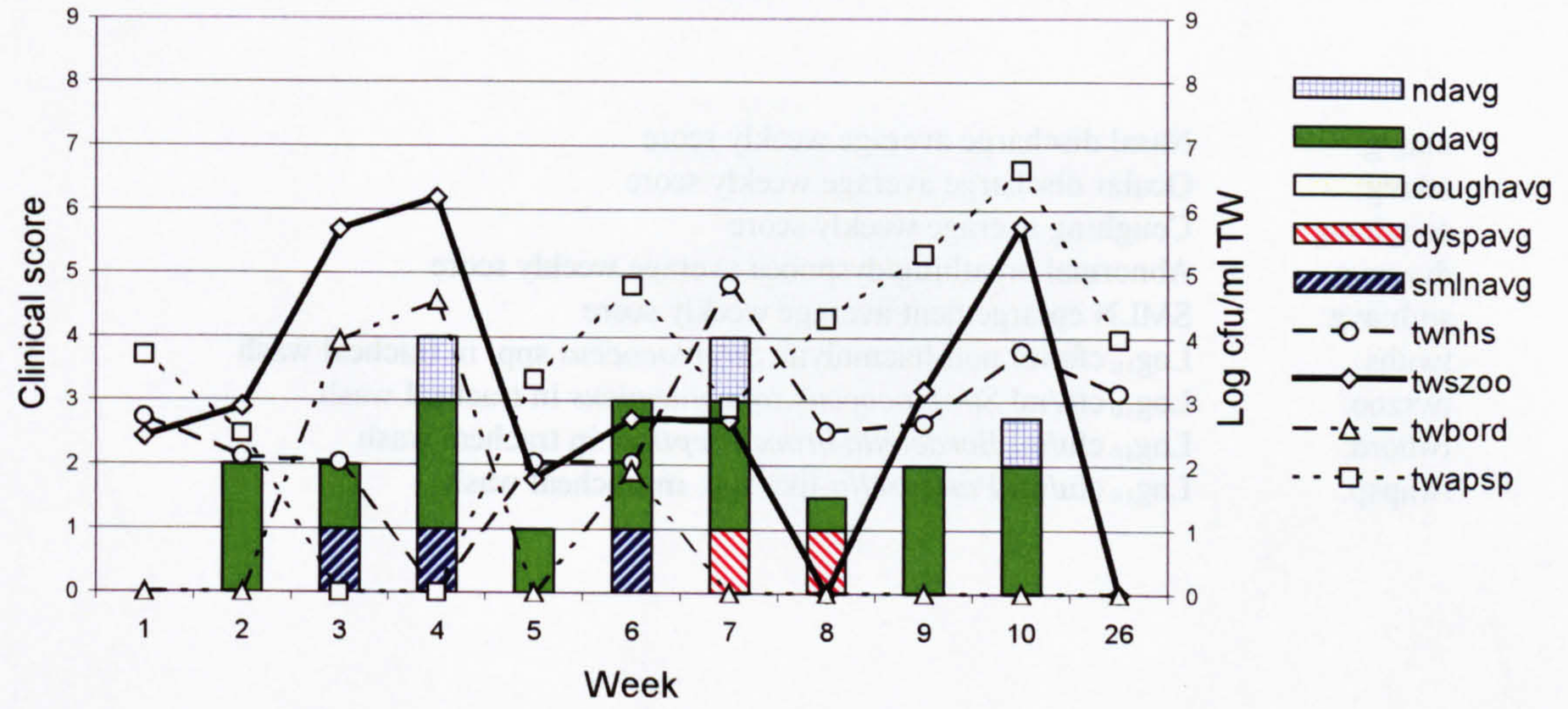
**Pony 25 (F2)**



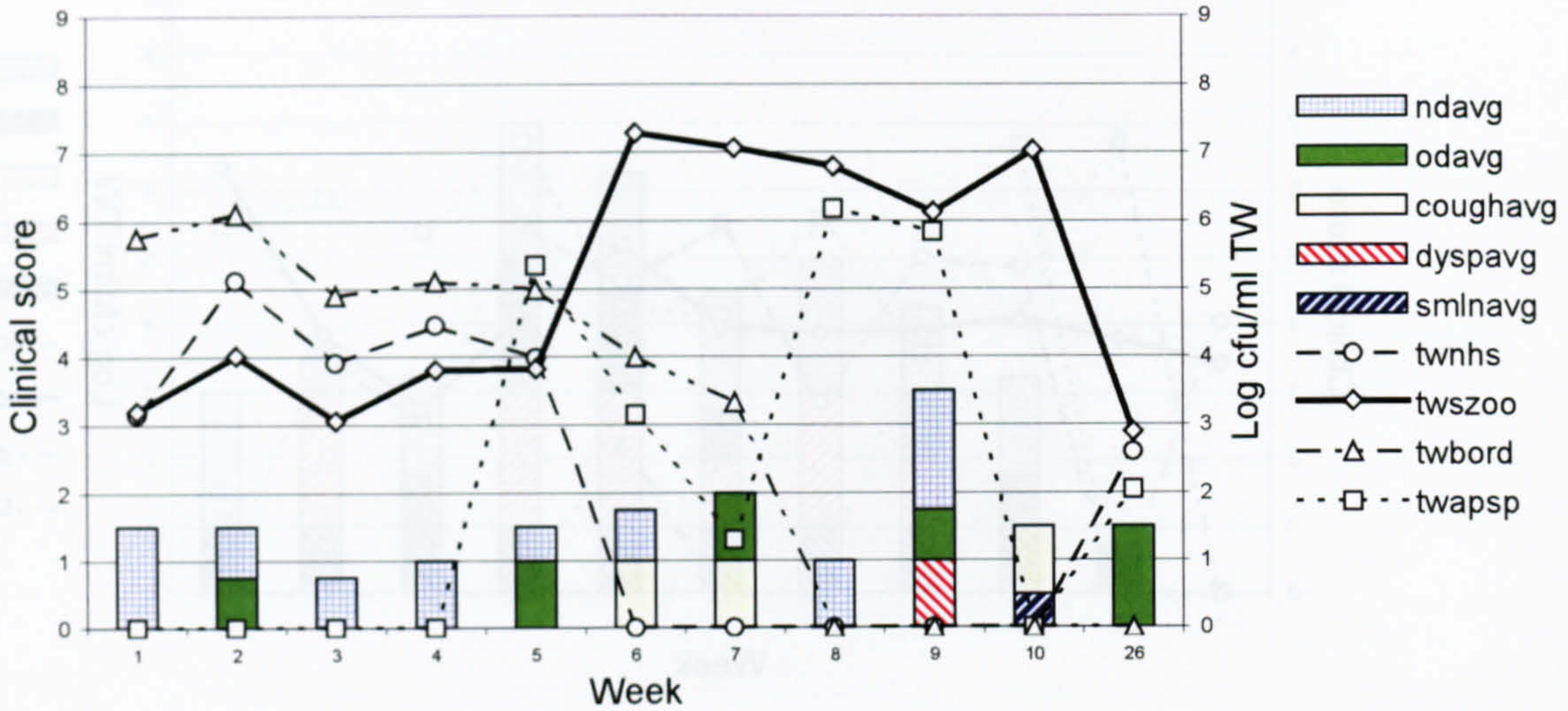

**Pony 26 (F2)**



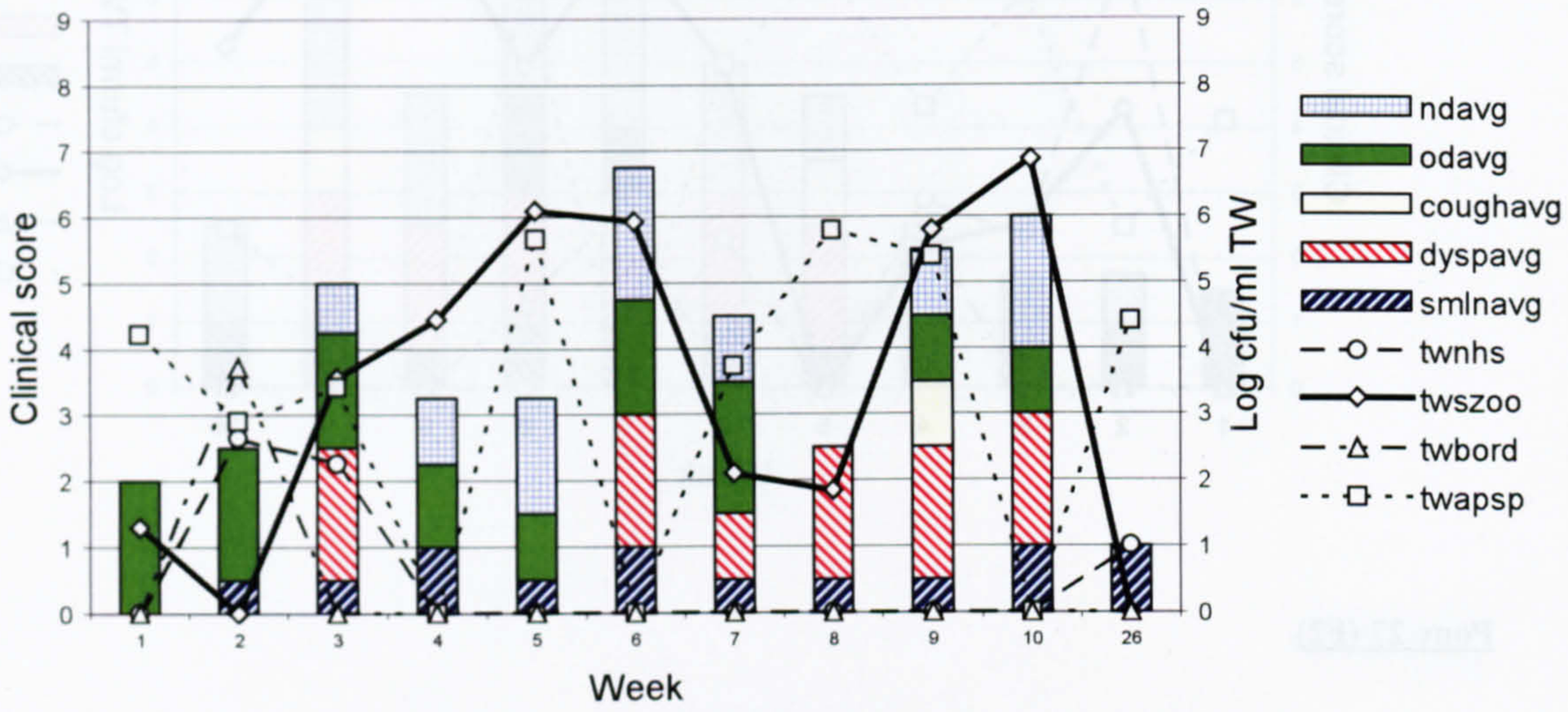

**Pony 27 (F2)**



**Pony 28 (F2)**




**Pony 29 (DF2)**




ndavg: Nasal discharge average weekly score
odavg: Ocular discharge average weekly score
coughavg: Coughing average weekly score
dyspavg: Abnormal breathing/dyspnoea average weekly score
smlnavg: SMLN enlargement average weekly score
twnhs: $Log_{10}$ cfu/ml non-haemolytic *Streptococcus* spp. in tracheal wash
twszoo: $Log_{10}$ cfu/ml *Streptococcus zooepidemicus* in tracheal wash
twbord: $Log_{10}$ cfu/ml *Bordetella bronchiseptica*. in tracheal wash
twapsp: $Log_{10}$ cfu/ml *Pasteurella*-like spp. in tracheal wash

**Table A2.15: Results and comparison of univariable linear and best fitting polynomial regressions of clinical and airway inflammation parameter scores with tracheal wash bacterial count data and autoregressive variables**

| Outcome var. *Regression type* | Explanatory Variable | Regression coefficient | Intercept | $R^2$ value (%) | P-value | Deviance benefit | P-value ($\chi^2$, 3 df) |
|---|---|---|---|---|---|---|---|
| **Clinical score** | | | | | | | |
| *Linear* | $\log_{10}$ cfu/ml total bacteria | 0.49 | -0.15 | 10.7 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml total bacteria]$^{0.5}$ | -0.37 | 1.94 | 13.2 | 0.511 | 8.93 | 0.030 |
| | [$\log_{10}$ cfu/ml total bacteria]$^3$ | 0.008 | | | <0.001 | | |
| *Linear* | $\log_{10}$ cfu/ml *S. zooepidemicus* | 0.44 | 0.85 | 18.2 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *S. zooepidemicus*]$^{0.5}$ | -0.38 | 1.76 | 20.0 | 0.009 | 6.99 | 0.072 |
| | [$\log_{10}$ cfu/ml *S. zooepidemicus*]$^1$ | 0.88 | | | <0.001 | | |
| *Linear* | $\log_{10}$ cfu/ml *A. equuli* | -0.08 | 2.40 | 0.2 | 0.444 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *A. equuli*]$^3$ | -0.05 | 2.41 | 0.8 | 0.125 | 1.99 | 0.574 |
| | [$\log_{10}$ cfu/ml *A. equuli*]$^3$×ln[x] | 0.03 | | | 0.114 | | |
| *Linear* | $\log_{10}$ cfu/ml *Pasteurella* spp. | 0.03 | 2.27 | 0.1 | 0.503 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *Pasteurella* spp.]$^1$ | -0.35 | 2.53 | 6.3 | <0.001 | 19.96 | <0.001 |
| | [$\log_{10}$ cfu/ml *Pasteurella* spp.]$^3$ | 0.01 | | | <0.001 | | |
| *Linear* | $\log_{10}$ cfu/ml *B. bronchiseptica* | -0.02 | 2.39 | 0.05 | 0.695 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *B. bronchiseptica*]$^{0.5}$ | 0.55 | 2.31 | 0.30 | 0.361 | 0.84 | 0.840 |
| | [$\log_{10}$ cfu/ml *B. bronchiseptica*]$^1$ | -0.25 | | | 0.331 | | |
| *Linear* | $\log_{10}$ cfu/ml non-haem. *Strep.* | -0.21 | 3.05 | 5.2 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml non-haem. *Strep.*]$^{0.5}$ | -1.35 | 3.78 | 15.8 | <0.001 | 37.21 | <0.001 |
| | [$\log_{10}$ cfu/ml non-haem. *Strep.*]$^3$ | 0.01 | | | <0.001 | | |
| *Linear* | Clinical score 1 week previously | 0.59 | 1.16 | 34.2 | <0.001 | | |
| *Polynomial* | [Clinical score 1 wk previously]$^2$ | 0.21 | 1.36 | 34.6 | <0.001 | 1.42 | 0.701 |
| | [Clinical score 1 wk previously]$^3$ | -0.02 | | | 0.001 | | |
| *Linear* | [Clinical score 2 weeks previously | 0.51 | 1.47 | 25.2 | <0.001 | | |
| *Polynomial* | [Clinical score 2 wks prev.]$^2$ | 0.34 | 1.39 | 26.2 | <0.001 | 3.03 | 0.387 |
| | [Clin. score 2 wks prev.]$^2$×$\log_n$[x] | -0.15 | | | <0.001 | | |
| *Linear* | Clinical score 3 weeks previously | 0.43 | 1.90 | 17.3 | <0.001 | | |
| *Polynomial* | [Clinical score 3 wks previously]$^2$ | 0.25 | 1.84 | 20.0 | <0.001 | 6.70 | 0.082 |
| | [Clinical score 3 wks previously]$^3$ | -0.03 | | | <0.001 | | |

Table A2.15 continued

| Outcome var. *Regression type* | Explanatory Variable | Regression coefficient | Intercept | $R^2$ value (%) | P-value | Deviance benefit | P-value ($\chi^2$, 3 df) |
|---|---|---|---|---|---|---|---|
| **CDNS score** | | | | | | | |
| *Linear* | $\log_{10}$ cfu/ml total bacteria | 0.45 | -0.81 | 12.7 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml total bacteria]$^3$ | -0.02 | 0.99 | 17.9 | 0.186 | 19.15 | <0.001 |
| | [$\log_{10}$ cfu/ml total bact.]$^3$×$\log_n$[x] | 0.01 | | | 0.067 | | |
| *Linear* | $\log_{10}$ cfu/ml *S. zooepidemicus* | 0.40 | 0.13 | 21.0 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *S. zooepidemicus*]$^{0.5}$ | -0.33 | 1.03 | 26.0 | 0.152 | 20.32 | <0.001 |
| | [$\log_{10}$ cfu/ml *S.* zooepidemicus]$^2$ | 0.07 | | | <0.001 | | |
| *Linear* | $\log_{10}$ cfu/ml *A. equuli* | -0.03 | 1.52 | 0.03 | 0.770 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *A.* equuli]$^3$ | -0.05 | 1.54 | 1.5 | 0.088 | 4.61 | 0.203 |
| | [$\log_{10}$ cfu/ml *A. equuli*]$^3$×$\log_n$[x] | 0.03 | | | 0.061 | | |
| *Linear* | $\log_{10}$ cfu/ml *Pasteurella* spp. | 0.03 | 1.42 | 0.2 | 0.434 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *Pasteurella* spp.]$^2$ | -0.13 | 1.61 | 7.4 | <0.001 | 23.42 | <0.001 |
| | [$\log_{10}$ cfu/ml *Pasteurella* spp.]$^3$ | 0.02 | | | <0.001 | | |
| *Linear* | $\log_{10}$ cfu/ml *B. bronchiseptica* | -0.02 | 1.53 | 0.09 | 0.600 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *B.* bronchiseptica]$^1$ | 0.04 | 1.52 | 0.12 | 0.842 | 0.10 | 0.992 |
| | [$\log_{10}$ cfu/ml *B. bronch.*]$^1$×$\log_n$[x] | -0.04 | | | 0.755 | | |
| *Linear* | $\log_{10}$ cfu/ml non-haem. *Strep.* | -0.25 | 2.30 | 9.9 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml non-haem. *Strep.*]$^{0.5}$ | -1.23 | 2.92 | 19.9 | <0.001 | 37.04 | <0.001 |
| | [$\log_{10}$ cfu/ml non-haem. *Strep.*]$^3$ | 0.01 | | | <0.001 | | |
| *Linear* | CDNS score 1 week previously | 0.66 | 0.62 | 41.2 | <0.001 | | |
| *Polynomial* | [CDNS score 1 wk previously]$^1$ | 0.55 | 0.52 | 41.3 | 0.001 | 0.42 | 0.936 |
| | [CDNS score 1 wk previously]$^2$ | 0.02 | | | 0.520 | | |
| *Linear* | CDNS score 2 weeks previously | 0.53 | 0.91 | 26.2 | <0.001 | | |
| *Polynomial* | [CDNS score 2 wks previously]$^2$ | 0.37 | 0.87 | 26.8 | <0.001 | 1.77 | 0.621 |
| | [CDNS score 2 wks prev.]$^3$×$\log_n$[x] | -0.17 | | | <0.001 | | |
| *Linear* | CDNS score 3 weeks previously | 0.41 | 1.23 | 15.0 | <0.001 | | |
| *Polynomial* | [CDNS score 3 wks previously]$^3$ | 0.14 | 1.25 | 18.6 | <0.001 | 8.65 | 0.034 |
| | [CDNS score 3 wks prev.]$^3$×$\log_n$[x] | -0.07 | | | <0.001 | | |
| **Airway inflammation score** | | | | | | | |
| *Linear* | $\log_{10}$ cfu/ml total bacteria | 0.80 | 2.06 | 26.4 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml total bacteria]$^{-2}$ | 0.001 | -2.26 | 27.7 | <0.001 | 5.35 | 0.148 |
| | [$\log_{10}$ cfu/ml total bacteria]$^{0.5}$ | 3.74 | | | <0.001 | | |
| *Linear* | $\log_{10}$ cfu/ml *S. zooepidemicus* | 0.58 | 4.23 | 27.9 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *S. zooepidemicus*]$^{-2}$ | 0.0002 | 1.97 | 28.4 | <0.001 | 2.17 | 0.538 |
| | [$\log_{10}$ cfu/ml *S. zooepidemicus*]$^{0.5}$ | 2.33 | | | <0.001 | | |
| *Linear* | $\log_{10}$ cfu/ml *A. equuli* | 0.12 | 6.15 | 0.4 | 0.278 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *A. equuli*]$^{-2}$ | -0.0001 | 6.26 | 0.7 | 0.725 | 0.93 | 0.818 |
| | [$\log_{10}$ cfu/ml *A. equuli*]$^3$ | 0.006 | | | 0.248 | | |
| *Linear* | $\log_{10}$ cfu/ml *Pasteurella* spp. | 0.17 | 5.71 | 4.0 | <0.001 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *Pasteurella* spp.]$^2$ | -0.12 | 5.93 | 8.0 | 0.053 | 13.47 | 0.004 |
| | [$\log_{10}$ cfu/ml *Past.* spp.]$^2$×$\log_n$[x] | 0.08 | | | 0.011 | | |
| *Linear* | $\log_{10}$ cfu/ml *B. bronchiseptica* | -0.02 | 6.23 | 0.04 | 0.720 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml *B.* bronchiseptica]$^3$ | -0.04 | 6.27 | 0.50 | 0.063 | 3.53 | 0.317 |
| | [$\log_{10}$ cfu/ml *B. bronch.*]$^2$×$\log_n$[x] | 0.02 | | | 0.059 | | |
| *Linear* | $\log_{10}$ cfu/ml non-haem. *Strep.* | -0.10 | 6.53 | 1.0 | 0.074 | | |
| *Polynomial* | [$\log_{10}$ cfu/ml non-haem. *Strep.*]$^{-1}$ | 0.09 | 4.28 | 9.8 | <0.001 | 29.00 | <0.001 |
| | $L_n$[$\log_{10}$ cfu/ml non-haem. *Strep.*] | 1.23 | | | <0.001 | | |
| *Linear* | Inflam. score 1 week previously | 0.46 | 3.45 | 22.7 | <0.001 | | |
| *Polynomial* | [Inflam. score 1 wk previously]$^{-0.5}$ | 13.1 | -11.6 | 26.2 | <0.001 | 11.89 | 0.008 |
| | [Inflam. score 1 wk previously]$^{0.5}$ | 4.82 | | | <0.001 | | |
| *Linear* | Inflam. score 2 weeks previously | 0.37 | 4.18 | 17.4 | <0.001 | | |
| *Polynomial* | [Inflam. score 2 wks previously]$^{-2}$ | -2.81 | 8.08 | 20.1 | <0.001 | 7.59 | 0.055 |
| | [Inflam. score 2 wks prev.]$^{-2}$×$\log_n$[x] | -36.1 | | | <0.001 | | |
| *Linear* | Inflam. score 3 weeks previously | 0.16 | 5.75 | 4.0 | <0.001 | | |
| *Polynomial* | [Inflam. score 3 wks previously]$^{-2}$ | 1.53 | 5.62 | 7.5 | 0.037 | 7.51 | 0.057 |
| | [Inflam. score 3 wks previously]$^2$ | 0.02 | | | <0.001 | | |

# Table A2.16: Summary of non-parametric analyses examining differences in clinical & airway inflammation outcomes for various binary/categorical explanatory variables

| Outcome variable | Explanatory variable | Non-parametric test | P-value |
|---|---|---|---|
| **Clinical score** | **Sex** | **Wilcoxon rank sum** | **0.0156** |
| | **Vaccine group** | **Kruskal-Wallis** | **0.0074** |
| | **Transferrin D** | **Wilcoxon rank sum** | **<0.0001** |
| | **Transferrin F2** | **Wilcoxon rank sum** | **<0.0001** |
| | Transferrin H1 | Wilcoxon rank sum | 0.67 |
| | Transferrin H2 | Wilcoxon rank sum | 0.63 |
| | Transferrin O | Wilcoxon rank sum | 0.79 |
| | Transferrin R | Wilcoxon rank sum | 0.70 |
| | Protease inhibitor I | Wilcoxon rank sum | 0.13 |
| | **Protease inhibitor L** | **Wilcoxon rank sum** | **0.0021** |
| | **Protease inhibitor L2** | **Wilcoxon rank sum** | **0.0105** |
| | Protease inhibitor R | Wilcoxon rank sum | 0.21 |
| | **Protease inhibitor S** | **Wilcoxon rank sum** | **0.0072** |
| | **NP *S. zooepidemicus*** | **Wilcoxon rank sum** | **<0.0001** |
| | **NP *Pasteurella* spp.** | **Wilcoxon rank sum** | **0.0530** |
| | NP *B. bronchiseptica* | Wilcoxon rank sum | 0.68 |
| | NP non-haemolytic *Streptococcus* spp. | Wilcoxon rank sum | 0.63 |
| | NP *Staphylococcus* spp. | Wilcoxon rank sum | 0.67 |
| **CDNS score** | **Sex** | **Wilcoxon rank sum** | **0.0062** |
| | **Vaccine group** | **Kruskal-Wallis** | **0.0056** |
| | **Transferrin D** | **Wilcoxon rank sum** | **<0.0001** |
| | **Transferrin F2** | **Wilcoxon rank sum** | **<0.0001** |
| | Transferrin H1 | Wilcoxon rank sum | 0.83 |
| | Transferrin H2 | Wilcoxon rank sum | 0.60 |
| | Transferrin O | Wilcoxon rank sum | 0.46 |
| | Transferrin R | Wilcoxon rank sum | 0.74 |
| | Protease inhibitor I | Wilcoxon rank sum | 0.17 |
| | **Protease inhibitor L** | **Wilcoxon rank sum** | **0.0014** |
| | **Protease inhibitor L2** | **Wilcoxon rank sum** | **0.0022** |
| | Protease inhibitor R | Wilcoxon rank sum | 0.96 |
| | Protease inhibitor S | Wilcoxon rank sum | 0.075 |
| | **NP *S. zooepidemicus*** | **Wilcoxon rank sum** | **<0.0001** |
| | NP *Pasteurella* spp. | Wilcoxon rank sum | 0.62 |
| | NP *B. bronchiseptica* | Wilcoxon rank sum | 0.74 |
| | NP non-haemolytic *Streptococcus* spp. | Wilcoxon rank sum | 0.32 |
| | NP *Staphylococcus* spp. | Wilcoxon rank sum | 0.38 |
| **Airway inflammation score** | **Sex** | **Wilcoxon rank sum** | **0.0019** |
| | Vaccine group | Kruskal-Wallis | 0.19 |
| | Transferrin D | Wilcoxon rank sum | 0.26 |
| | **Transferrin F2** | **Wilcoxon rank sum** | **0.0059** |
| | **Transferrin H1** | **Wilcoxon rank sum** | **0.0242** |
| | Transferrin H2 | Wilcoxon rank sum | 0.39 |
| | Transferrin O | Wilcoxon rank sum | 0.18 |
| | Transferrin R | Wilcoxon rank sum | 0.07 |
| | Protease inhibitor I | Wilcoxon rank sum | 0.46 |
| | **Protease inhibitor L** | **Wilcoxon rank sum** | **0.0423** |
| | **Protease inhibitor L2** | **Wilcoxon rank sum** | **0.0534** |
| | Protease inhibitor R | Wilcoxon rank sum | 0.49 |
| | Protease inhibitor S | Wilcoxon rank sum | 0.35 |
| | **NP *S. zooepidemicus*** | **Wilcoxon rank sum** | **0.0073** |
| | **NP *Pasteurella* spp.** | **Wilcoxon rank sum** | **0.0484** |
| | NP *B. bronchiseptica* | Wilcoxon rank sum | 0.29 |
| | NP non-haemolytic *Streptococcus* spp. | Wilcoxon rank sum | 0.26 |
| | NP *Staphylococcus* spp. | Wilcoxon rank sum | 0.24 |

**Figure A2.7: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for clinical score, including transferrin D haplotype (Model 4a)**

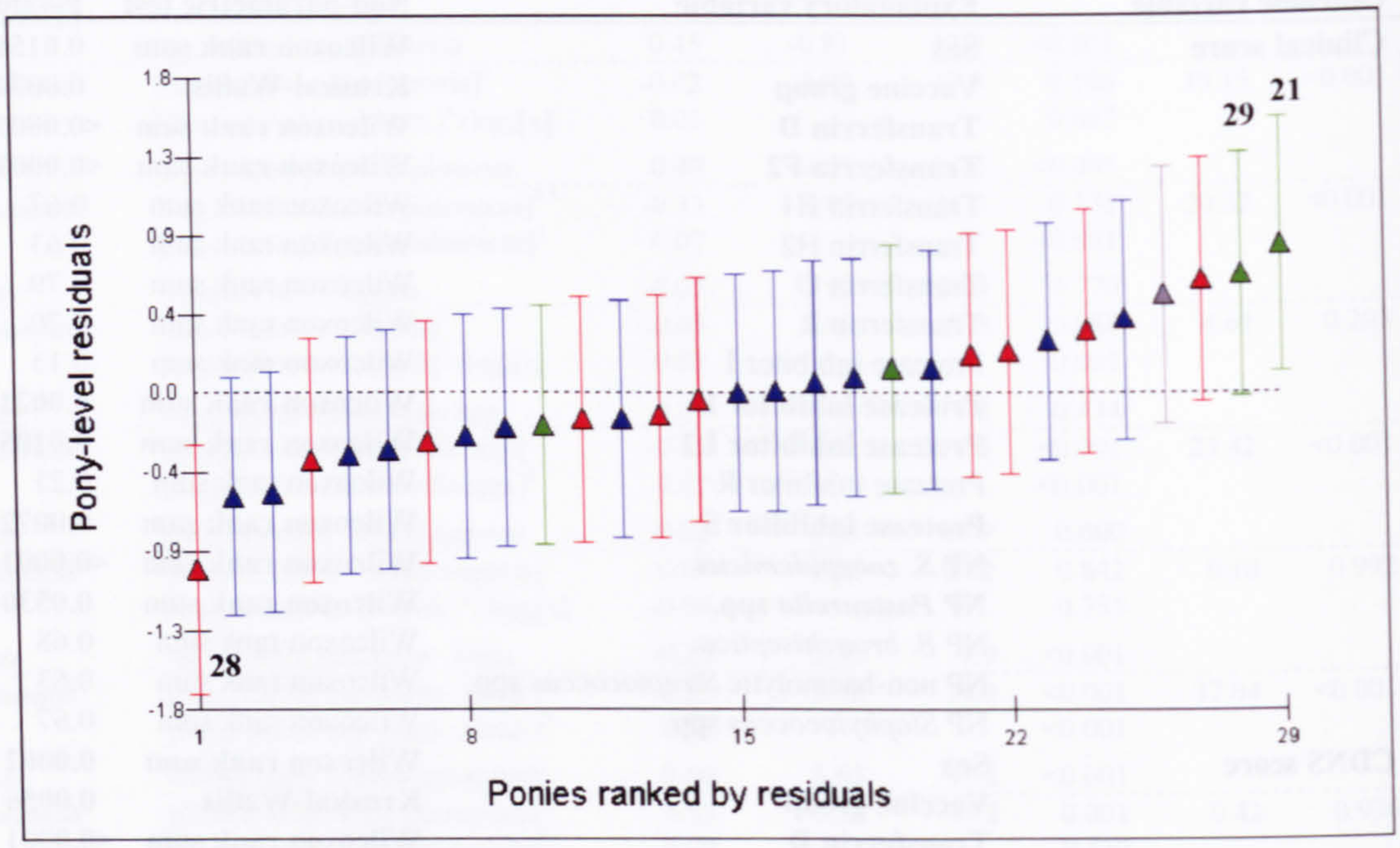
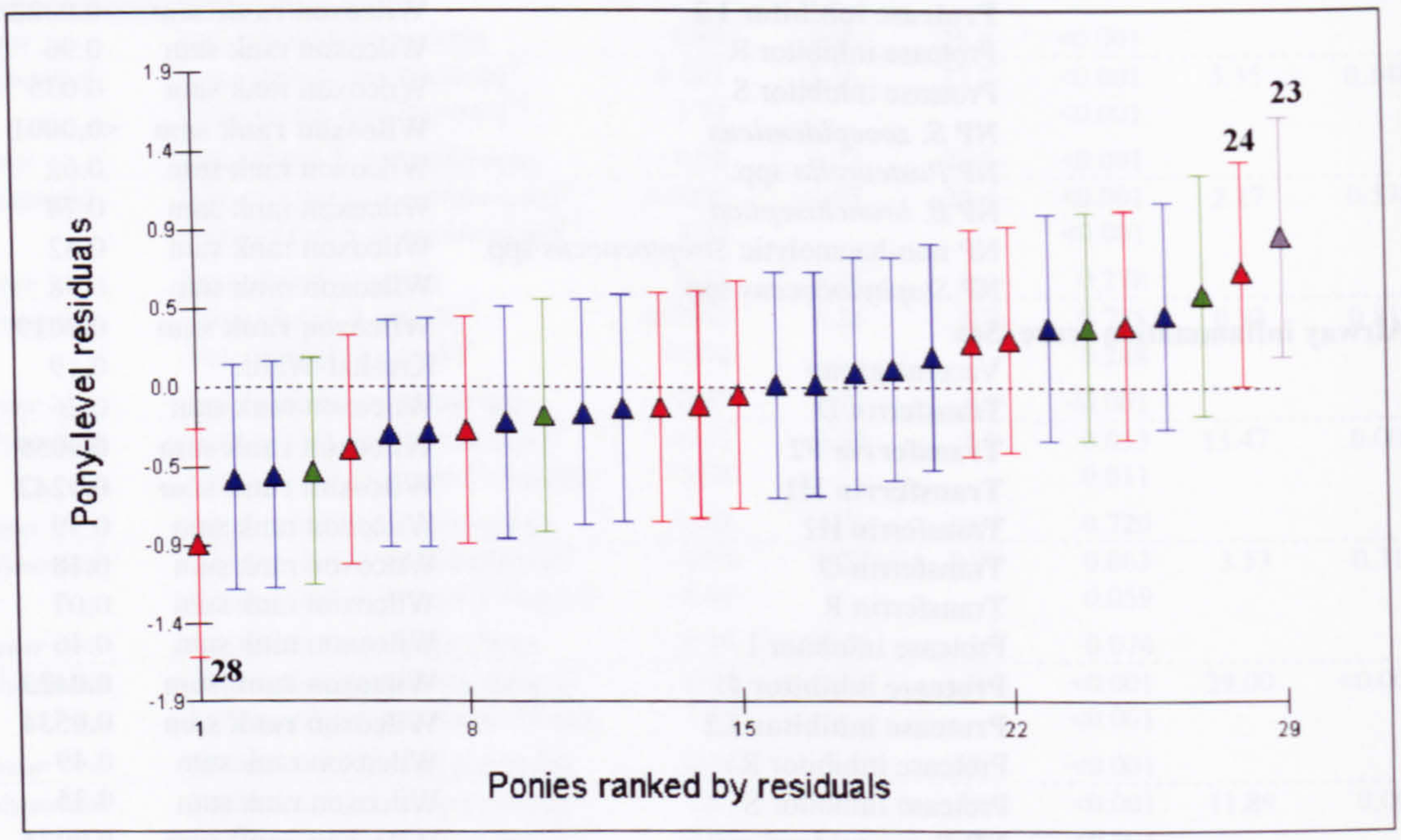



**Figure A2.8: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for clinical score, including transferrin F2 haplotype (Model 4b)**




Note: Colour scheme represents the transferrin D/F2 haplotype status of each pony light blue: transferrin D haplotype (n=14), red: transferrin F2 haplotype (n=10), ight green: transferrin DF2 haplotype (n=4) & grey: other transferrin haplotype (n=1)

**Figure A2.9: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for CDNS score, including transferrin D haplotype (Model 4a)**

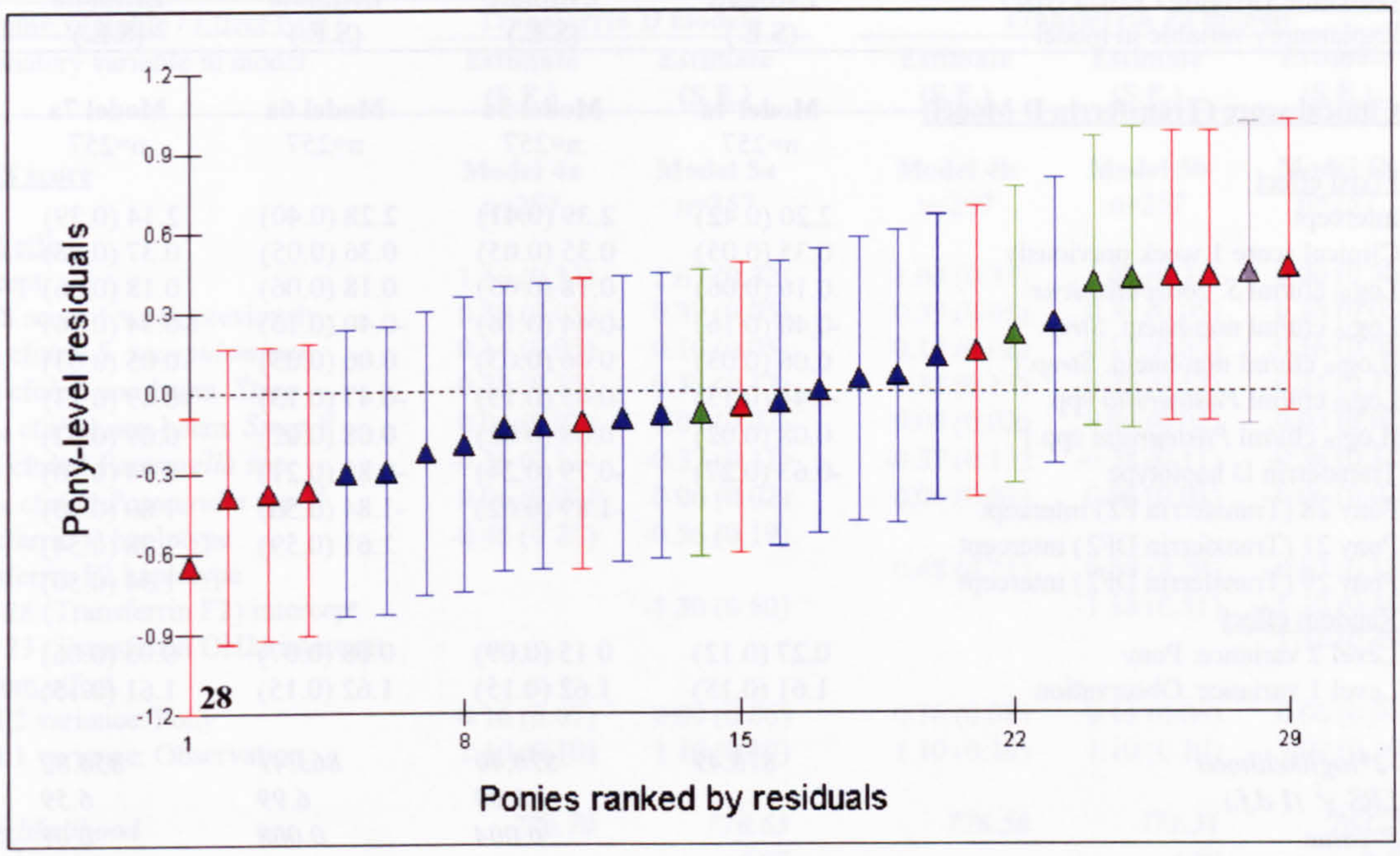
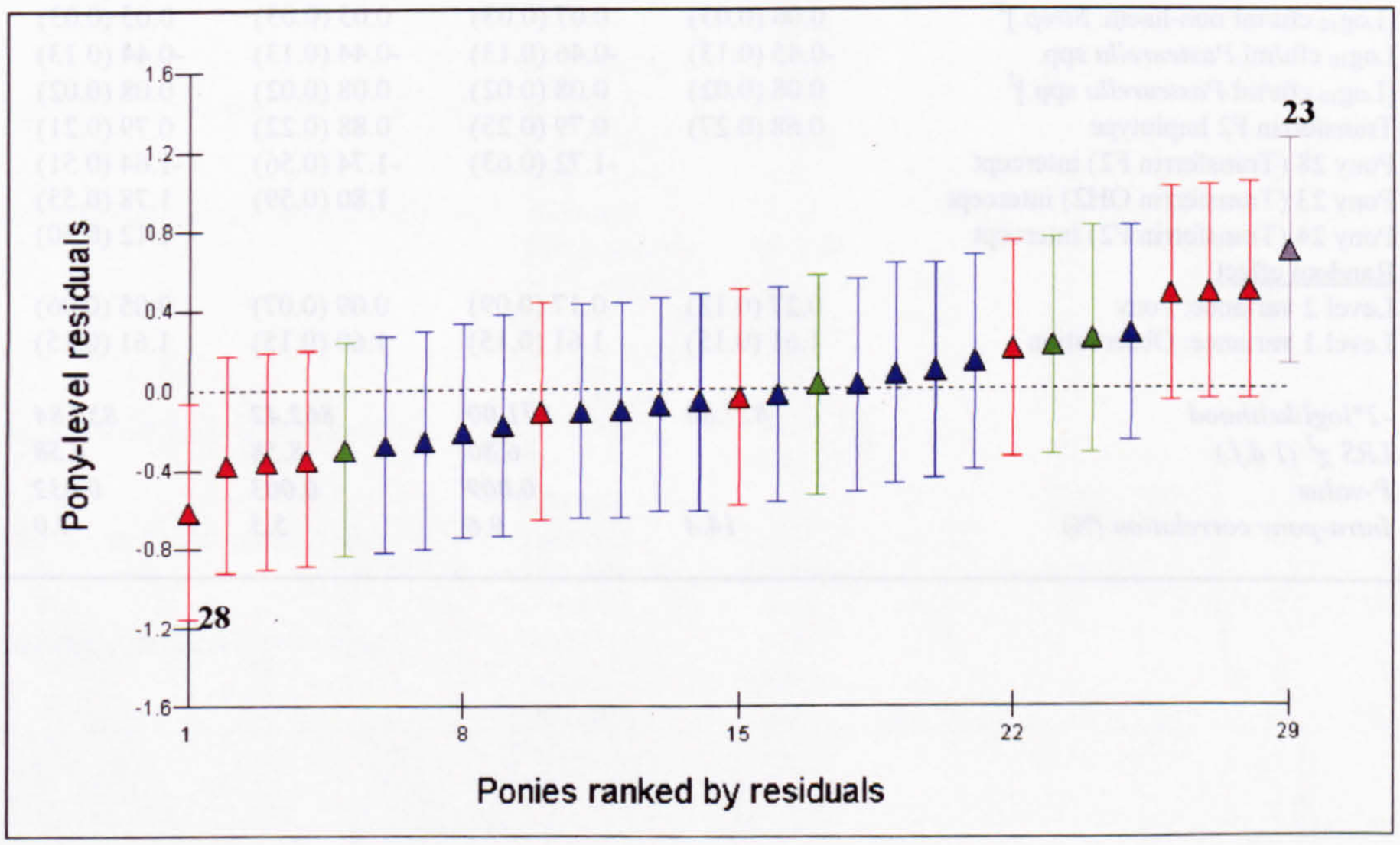



**Figure A2.10: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for CDNS score, including transferrin F2 haplotype (Model 4b)**




Note: Colour scheme represents the transferrin D/F2 haplotype status of each pony light blue: transferrin D haplotype (n=14), red: transferrin F2 haplotype (n=10), light green: transferrin DF2 haplotype (n=4) & grey: other transferrin haplotype (n=1)

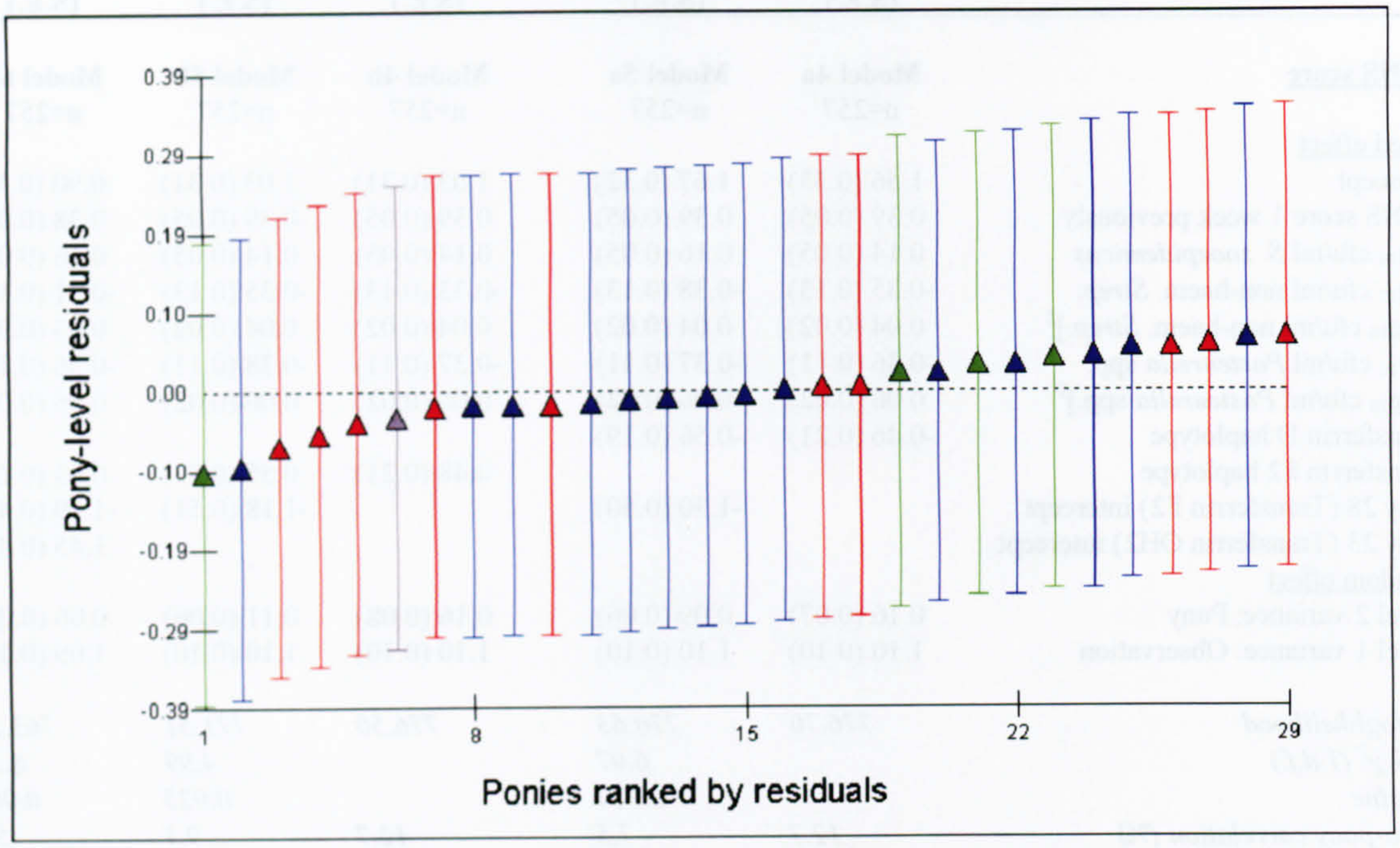
# Table A2.17: Summary of multilevel linear regression modelling of clinical score with sequential exclusion from random effects components of ponies with largest value residuals

| Outcome variable / Effect type / Explanatory variable in model | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) |
|---|---|---|---|---|
| **Clinical score (Transferrin D Model)** | **Model 4a n=257** | **Model 5a n=257** | **Model 6a n=257** | **Model 7a n=257** |
| Fixed effect | | | | |
| Intercept | 2.20 (0.42) | 2.39 (0.41) | 2.28 (0.40) | 2.14 (0.39) |
| Clinical score 1 week previously | 0.35 (0.05) | 0.35 (0.05) | 0.36 (0.05) | 0.37 (0.05) |
| $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.16 (0.06) | 0.18 (0.06) | 0.18 (0.06) | 0.18 (0.06) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.40 (0.16) | -0.44 (0.16) | -0.40 (0.16) | -0.34 (0.16) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.06 (0.03) | 0.06 (0.03) | 0.06 (0.03) | 0.05 (0.03) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.44 (0.13) | -0.45 (0.13) | -0.47 (0.13) | -0.49 (0.13) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.08 (0.02) | 0.08 (0.02) | 0.08 (0.02) | 0.09 (0.02) |
| Transferrin D haplotype | -0.63 (0.27) | -0.79 (0.24) | -0.85 (0.21) | -0.94 (0.20) |
| Pony 28 (Transferrin F2) intercept | | -1.89 (0.62) | -1.84 (0.56) | -1.80 (0.50) |
| Pony 21 (Transferrin DF2) intercept | | | 1.61 (0.59) | 1.78 (0.54) |
| Pony 29 (Transferrin DF2) intercept | | | | 1.34 (0.50) |
| Random effect | | | | |
| Level 2 variance: Pony | 0.27 (0.12) | 0.15 (0.09) | 0.08 (0.07) | 0.03 (0.06) |
| Level 1 variance: Observation | 1.61 (0.15) | 1.62 (0.15) | 1.62 (0.15) | 1.61 (0.15) |
| *-2*loglikelihood* | *878.49* | *870.40* | *863.41* | *856.82* |
| *LRS $\chi^2$ (1 d.f.)* | | *8.09* | *6.99* | *6.59* |
| *P-value* | | *0.004* | *0.008* | *0.01* |
| *Intra-pony correlation (%)* | *14.4* | *8.5* | *4.7* | *1.8* |
| **Clinical score (Transferrin F2 Model)** | **Model 4b n=257** | **Model 5b n=257** | **Model 6b n=257** | **Model 7b n=257** |
| Fixed effect | | | | |
| Intercept | 1.46 (0.39) | 1.47 (0.38) | 1.33 (0.37) | 1.40 (0.37) |
| Clinical score 1 week previously | 0.35 (0.05) | 0.35 (0.05) | 0.34 (0.05) | 0.34 (0.05) |
| $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.15 (0.06) | 0.16 (0.06) | 0.17 (0.06) | 0.16 (0.06) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.38 (0.16) | -0.41 (0.16) | -0.34 (0.16) | -0.37 (0.16) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.06 (0.03) | 0.07 (0.03) | 0.05 (0.03) | 0.05 (0.03) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.45 (0.13) | -0.46 (0.13) | -0.44 (0.13) | -0.44 (0.13) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.08 (0.02) | 0.08 (0.02) | 0.08 (0.02) | 0.08 (0.02) |
| Transferrin F2 haplotype | 0.68 (0.27) | 0.79 (0.25) | 0.88 (0.22) | 0.79 (0.21) |
| Pony 28 (Transferrin F2) intercept | | -1.72 (0.63) | -1.74 (0.56) | -1.64 (0.51) |
| Pony 23 (Transferrin OH2) intercept | | | 1.80 (0.59) | 1.78 (0.55) |
| Pony 24 (Transferrin F2) intercept | | | | 1.12 (0.50) |
| Random effect | | | | |
| Level 2 variance: Pony | 0.27 (0.12) | 0.17 (0.09) | 0.09 (0.07) | 0.05 (0.06) |
| Level 1 variance: Observation | 1.61 (0.15) | 1.61 (0.15) | 1.60 (0.15) | 1.61 (0.15) |
| *-2*loglikelihood* | *877.80* | *871.00* | *862.42* | *857.84* |
| *LRS $\chi^2$ (1 d.f.)* | | *6.80* | *8.58* | *4.58* |
| *P-value* | | *0.009* | *0.003* | *0.032* |
| *Intra-pony correlation (%)* | *14.4* | *9.6* | *5.3* | *3.0* |

# Table A2.18: Summary of multilevel linear regression modelling of CDNS score with sequential exclusion from random effects components of ponies with largest value residuals

| Outcome variable / Effect type / Explanatory variable in model | Transferrin D models | | Transferrin F2 models | | |
|---|---|---|---|---|---|
| | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) |
| **CDNS score** | **Model 4a n=257** | **Model 5a n=257** | **Model 4b n=257** | **Model 5b n=257** | **Model 6b n=257** |
| Fixed effect | | | | | |
| Intercept | 1.56 (0.33) | 1.67 (0.32) | 1.03 (0.31) | 1.03 (0.31) | 0.90 (0.30) |
| CDNS score 1 week previously | 0.39 (0.05) | 0.39 (0.05) | 0.39 (0.05) | 0.39 (0.05) | 0.38 (0.05) |
| $Log_{10}$ cfu/ml *S. zooepidemicus* | 0.14 (0.05) | 0.16 (0.05) | 0.14 (0.05) | 0.14 (0.05) | 0.16 (0.05) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.35 (0.13) | -0.38 (0.13) | -0.33 (0.13) | -0.35 (0.13) | -0.31 (0.13) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.04 (0.02) | 0.04 (0.02) | 0.04 (0.02) | 0.04 (0.02) | 0.03 (0.02) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.36 (0.11) | -0.37 (0.11) | -0.37 (0.11) | -0.38 (0.11) | -0.36 (0.11) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp.$]^2$ | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) |
| Transferrin D haplotype | -0.46 (0.21) | -0.56 (0.19) | | | |
| Transferrin F2 haplotype | | | 0.48 (0.21) | 0.55 (0.20) | 0.63 (0.18) |
| Pony 28 (Transferrin F2) intercept | | -1.30 (0.50) | | -1.18 (0.51) | -1.19 (0.45) |
| Pony 23 (Transferrin OH2) intercept | | | | | 1.45 (0.49) |
| Random effect | | | | | |
| Level 2 variance: Pony | 0.16 (0.07) | 0.09 (0.06) | 0.16 (0.08) | 0.11 (0.06) | 0.06 (0.05) |
| Level 1 variance: Observation | 1.10 (0.10) | 1.10 (0.10) | 1.10 (0.10) | 1.10 (0.10) | 1.09 (0.10) |
| *-2*loglikelihood* | *776.70* | *770.63* | *776.50* | *771.51* | *763.21* |
| *LRS $\chi^2$ (1 d.f.)* | | *6.07* | | *4.99* | *8.30* |
| *P-value* | | *0.014* | | *0.025* | *0.004* |
| *Intra-pony correlation (%)* | *12.7* | *7.5* | *12.7* | *9.1* | *5.2* |

**Figure A2.11: Caterpillar plot of ranked pony level residuals (± 95% CI) for multilevel linear regression model for airway inflammation score excluding transferrin H1 haplotype (Model 2, Table 8.2)**




Note: Colour scheme represents the transferrin D/F2 haplotype status of each pony light blue: transferrin D haplotype (n=14), red: transferrin F2 haplotype (n=10), light green: transferrin DF2 haplotype (n=4) & grey: other transferrin haplotype (n=1)

# Table A2.19a: Results of univariable ordinary logistic regression (OLR) analyses of the risk of nasal discharge with different explanatory variables

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = nasal discharge* | | | | | | |
| TW $Log_{10}$fu/ml total bacteria | Intercept | -2.06 | 0.54 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.38 | 0.10 | 1.46 | 1.20 – 1.78 | <0.001 |
| TW $Log_{10}$cfu/ml total *S. zooepidemicus* | Intercept | -1.43 | 0.28 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.39 | 0.07 | 1.48 | 1.28 – 1.71 | <0.001 |
| TW *S. zooepidemicus* (categorical) | Intercept | -0.54 | 0.14 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | 0.50 | 0.31 | 1.65 | 0.90 – 3.03 | 0.108 |
| | $10^5$-$10^6$ cfu/ml | 2.33 | 0.56 | 10.3 | 3.43 – 30.7 | <0.001 |
| | >$10^6$ cfu/ml | 2.62 | 0.63 | 13.7 | 3.99 – 47.0 | <0.001 |
| TW $Log_{10}$cfu/ml *A. equuli* | Intercept | -0.05 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.09 | 0.11 | 0.92 | 0.74 – 1.14 | 0.441 |
| TW *A. equuli* (categorical) | Intercept | -0.02 | 0.13 | | | |
| | Not isolated | referet | | | | |
| | <$10^2$ cfu/ml | -0.33 | 0.37 | 0.72 | 0.35 – 1.48 | 0.370 |
| | ≥$10^2$ cfu/ml | -0.28 | 0.37 | 0.75 | 0.36 – 1.57 | 0.453 |
| TW $Log_{10}$cfu/ml *Pasteurella* spp. | Intercept | -0.06 | 0.18 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.01 | 0.05 | 0.99 | 0.90 – 1.09 | 0.840 |
| TW *Pasteurella* spp. (categorical) | Intercept | -0.15 | 0.15 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | 0.44 | 0.32 | 0.64 | 0.35 – 1.20 | 0.162 |
| | $10^5$-$10^6$ cfu/ml | 0.43 | 0.31 | 1.54 | 0.84 – 2.82 | 0.161 |
| | >$10^6$ cfu/ml | 0.77 | 0.46 | 2.17 | 0.88 – 5.37 | 0.094 |
| TW $Log_{10}$cfu/ml *B. bronchiseptica* | Intercept | -0.15 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.05 | 0.05 | 1.05 | 0.95 – 1.16 | 0.366 |
| TW *B. bronchiseptica* (categorical) | Intercept | -0.15 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | 0.34 | 0.39 | 1.40 | 0.66 – 2.99 | 0.378 |
| | $10^3$-$10^5$ cfu/ml | -0.10 | 0.42 | 0.91 | 0.40 – 2.09 | 0.822 |
| | >$10^5$ cfu/ml | 0.25 | 0.35 | 1.29 | 0.64 – 2.56 | 0.476 |
| TW $Log_{10}$cfu/ml non-haemolytic *Strep.* spp. | Intercept | 0.60 | 0.22 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.22 | 0.06 | 0.80 | 0.72 – 0.90 | <0.001 |
| TW non-haemolytic *Strep.* spp. (categorical) | Intercept | 1.12 | 0.29 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | -1.48 | 0.40 | 0.23 | 0.10 – 0.49 | <0.001 |
| | $10^3$-$10^4$ cfu/ml | -1.63 | 0.39 | 0.20 | 0.09 – 0.42 | <0.001 |
| | $10^4$-$10^5$ cfu/ml | -1.65 | 0.39 | 0.19 | 0.09 – 0.41 | <0.001 |
| | >$10^5$ cfu/ml | -1.27 | 0.38 | 0.28 | 0.13 – 0.59 | 0.001 |
| Nasal discharge 1 week previously | Intercept | -0.57 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 1.01 | 0.26 | 2.75 | 1.67 – 4.54 | <0.001 |
| Nasal discharge 2 weeks previously | Intercept | -0.37 | 0.19 | | | |
| | Absent | referent | | | | |
| | Present | 0.73 | 0.28 | 2.08 | 1.23 – 3.51 | 0.006 |
| Nasal discharge 3 weeks previously | Intercept | -0.41 | 0.19 | | | |
| | Absent | referent | | | | |
| | Present | 1.25 | 0.30 | 3.48 | 1.94 – 6.25 | <0.001 |

Table A2.19a continued

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = nasal discharge* | | | | | | |
| Sex | Intercept | 0.11 | 0.16 | | | |
| | Female | referent | | | | |
| | Male | -0.45 | 0.23 | 0.64 | 0.41 – 0.99 | 0.046 |
| Vaccine group | Intercept | -0.21 | 0.18 | | | |
| | Vaccine | referent | | | | |
| | Placebo | 0.03 | 0.25 | 1.03 | 0.63 – 1.67 | 0.902 |
| | Late intro | 0.54 | 0.32 | 1.72 | 0.91 – 3.25 | 0.094 |
| Transferrin D haplotype | Intercept | 0.22 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | -0.52 | 0.23 | 0.59 | 0.38 – 0.94 | 0.025 |
| Transferrin F2 haplotype | Intercept | -0.48 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | 0.77 | 0.23 | 2.16 | 1.38 – 3.38 | 0.001 |
| Transferrin H1 haplotype | Intercept | -0.10 | 0.12 | | | |
| | Absent | referent | | | | |
| | Present | -0.08 | 0.37 | 0.92 | 0.45 – 1.89 | 0.819 |
| Transferrin H2 haplotype | Intercept | -0.08 | 0.12 | | | |
| | Absent | referent | | | | |
| | Present | -0.19 | 0.33 | 0.83 | 0.43 – 1.56 | 0.553 |
| Transferrin O haplotype | Intercept | -0.09 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | -0.05 | 0.24 | 0.95 | 0.59 – 1.53 | 0.834 |
| Transferrin R haplotype | Intercept | -0.09 | 0.12 | | | |
| | Absent | referent | | | | |
| | Present | -0.09 | 0.33 | 0.92 | 0.48 – 1.74 | 0.788 |
| Protease inhibitor I haplotype | Intercept | -0.21 | 0.15 | | | |
| | Absent | referent | | | | |
| | Present | 0.22 | 0.23 | 1.25 | 0.80 – 1.94 | 0.331 |
| Protease inhibitor L haplotype | Intercept | 0.56 | 0.36 | | | |
| | Absent | referent | | | | |
| | Present | -0.74 | 0.38 | 0.48 | 0.23 – 1.00 | 0.051 |
| Protease inhibitor L2 haplotype | Intercept | -0.16 | 0.11 | | | |
| | Absent | referent | | | | |
| | Present | 1.66 | 0.79 | 5.26 | 1.11 – 24.7 | 0.036 |
| Protease inhibitor R haplotype | Intercept | -0.12 | 0.11 | | | |
| | Absent | referent | | | | |
| | Present | 0.30 | 0.62 | 1.35 | 0.40 – 4.51 | 0.627 |
| Protease inhibitor S haplotype | Intercept | -0.11 | 0.12 | | | |
| | Absent | referent | | | | |
| | Present | 0.17 | 0.30 | 1.19 | 0.66 – 2.13 | 0.560 |
| NP *S. zooepidemicus* | Intercept | -0.73 | 0.38 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.71 | 0.36 | 2.03 | 1.00 – 4.10 | 0.048 |
| NP *Pasteurella* spp. | Intercept | 0.14 | 0.38 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.27 | 0.40 | 0.76 | 0.35 – 1.65 | 0.490 |
| NP *B. Bronchiseptica* | Intercept | -0.19 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.35 | 0.27 | 1.42 | 0.84 – 2.41 | 0.188 |
| NP non-haemolytic *Streptococcus* spp. | Intercept | 0.33 | 0.36 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.48 | 0.38 | 0.62 | 0.29 – 1.31 | 0.211 |
| NP *Staphylococcus* spp. | Intercept | 0.25 | 0.36 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.40 | 0.38 | 0.67 | 0.32 – 1.40 | 0.289 |

# Table A2.19b: Results of univariable ordinary logistic regression (OLR) analyses of the risk of ocular discharge with different explanatory variables

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = ocular discharge* | | | | | | |
| TW $Log_{10}$ cfu/ml total bacteria | Intercept | -2.41 | 0.64 | | | |
| | Not isolated | refernt | | | | |
| | Log cfu/ml$^{-1}$ | 0.22 | 0.12 | 1.25 | 0.99 – 1.57 | 0.058 |
| TW $Log_{10}$ cfu/ml total *S. zooepidemicus* | Intercept | -1.58 | 0.30 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.10 | 0.08 | 1.10 | 0.95 – 1.28 | 0.209 |
| TW *S. zooepidemicus* (categorical) | Intercept | -1.19 | 0.16 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | -0.54 | 0.42 | 0.59 | 0.26 – 1.33 | 0.199 |
| | $10^5$-$10^6$ cfu/ml | -0.33 | 0.52 | 0.72 | 0.26 – 1.98 | 0.520 |
| | >$10^6$ cfu/ml | 0.50 | 0.44 | 1.65 | 0.69 – 3.90 | 0.258 |
| TW $Log_{10}$ cfu/ml *A. equuli* | Intercept | -1.26 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.01 | 0.13 | 1.01 | 0.79 – 1.31 | 0.910 |
| TW *A. equuli* (categorical) | Intercept | -1.27 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | <$10^2$ cfu/ml | -0.08 | 0.45 | 0.93 | 0.38 – 2.24 | 0.866 |
| | ≥$10^2$ cfu/ml | 0.29 | 0.42 | 1.34 | 0.59 – 3.05 | 0.486 |
| TW $Log_{10}$ cfu/ml *Pasteurella* spp. | Intercept | -1.12 | 0.21 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.04 | 0.06 | 0.96 | 0.85 – 1.07 | 0.452 |
| TW *Pasteurella* spp. (categorical) | Intercept | -1.18 | 0.18 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | -0.12 | 0.37 | 0.89 | 0.43 – 1.84 | 0.752 |
| | $10^5$-$10^6$ cfu/ml | -0.23 | 0.38 | 0.80 | 0.38 – 1.68 | 0.551 |
| | >$10^6$ cfu/ml | -0.10 | 0.54 | 0.91 | 0.32 – 2.59 | 0.854 |
| TW $Log_{10}$ cfu/ml *B. bronchiseptica* | Intercept | -1.41 | 0.16 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.12 | 0.06 | 1.12 | 1.00 – 1.26 | 0.052 |
| TW *B. bronchiseptica* (categorical) | Intercept | -1.44 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | 0.55 | 0.43 | 1.73 | 0.74 – 4.04 | 0.202 |
| | $10^3$-$10^5$ cfu/ml | 0.87 | 0.45 | 2.38 | 0.99 – 5.77 | 0.054 |
| | >$10^5$ cfu/ml | 0.41 | 0.41 | 1.51 | 0.68 – 3.36 | 0.308 |
| TW $Log_{10}$ cfu/ml non-haemolytic *Strep.* spp. | Intercept | -1.41 | 0.27 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.05 | 0.07 | 1.05 | 0.91 – 1.20 | 0.497 |
| TW non-haemolytic *Strep.* spp. (categorical) | Intercept | -1.39 | 0.31 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | 0.19 | 0.44 | 1.21 | 0.51 – 2.88 | 0.668 |
| | $10^3$-$10^4$ cfu/ml | -0.19 | 0.45 | 0.83 | 0.34 – 2.02 | 0.682 |
| | $10^4$-$10^5$ cfu/ml | 0.35 | 0.42 | 1.42 | 0.62 – 3.22 | 0.406 |
| | >$10^5$ cfu/ml | 0.27 | 0.42 | 1.31 | 0.57 – 2.99 | 0.528 |
| Ocular discharge 1 week previously | Intercept | -0.91 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | -0.23 | 0.33 | 0.79 | 0.92 – 1.51 | 0.483 |
| Ocular discharge 2 weeks previously | Intercept | -1.12 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 0.09 | 0.34 | 1.09 | 0.56 – 2.13 | 0.796 |
| Ocular discharge 3 weeks previously | Intercept | -0.97 | 0.19 | | | |
| | Absent | referent | | | | |
| | Present | -0.57 | 0.39 | 0.56 | 0.26 – 1.22 | 0.146 |

Table A2.19b continued

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = ocular discharge* | | | | | | |
| Sex | Intercept | -1.46 | 0.20 | | | |
| | Female | referent | | | | |
| | Male | 0.45 | 0.27 | 1.57 | 0.92 – 2.66 | 0.096 |
| Vaccine group | Intercept | -1.27 | 0.21 | | | |
| | Vaccine | referent | | | | |
| | Placebo | -0.09 | 0.30 | 0.91 | 0.51 – 1.65 | 0.763 |
| | Late intro | 0.38 | 0.36 | 1.46 | 0.71 – 2.97 | 0.301 |
| Transferrin D haplotype | Intercept | -1.20 | 0.22 | | | |
| | Absent | referent | | | | |
| | Present | -0.05 | 0.28 | 0.95 | 0.55 – 1.63 | 0.849 |
| Transferrin F2 haplotype | Intercept | -1.24 | 0.19 | | | |
| | Absent | referent | | | | |
| | Present | 0.02 | 0.27 | 1.02 | 0.60 – 1.72 | 0.948 |
| Transferrin H1 haplotype | Intercept | -1.22 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | -0.09 | 0.45 | 0.92 | 0.38 – 2.21 | 0.844 |
| Transferrin H2 haplotype | Intercept | -1.23 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | 0.01 | 0.39 | 1.01 | 0.47 – 2.16 | 0.979 |
| Transferrin O haplotype | Intercept | -1.17 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | -0.20 | 0.30 | 0.82 | 0.46 – 1.46 | 0.498 |
| Transferrin R haplotype | Intercept | -1.21 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | -0.14 | 0.40 | 0.87 | 0.39 – 1.90 | 0.718 |
| Protease inhibitor I haplotype | Intercept | -1.22 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | -0.02 | 0.27 | 0.98 | 0.58 – 1.66 | 0.941 |
| Protease inhibitor L haplotype | Intercept | -0.98 | 0.39 | | | |
| | Absent | referent | | | | |
| | Present | -0.28 | 0.42 | 0.75 | 0.33 – 1.70 | 0.496 |
| Protease inhibitor L2 haplotype | Intercept | -1.24 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | 0.26 | 0.69 | 1.30 | 0.34 – 5.03 | 0.705 |
| Protease inhibitor R haplotype | Intercept | -1.24 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | 0.26 | 0.69 | 1.30 | 0.34 – 5.03 | 0.705 |
| Protease inhibitor S haplotype | Intercept | -1.30 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | 0.31 | 0.34 | 1.36 | 0.70 – 2.64 | 0.360 |
| NP *S. zooepidemicus* | Intercept | -1.73 | 0.44 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.56 | 0.46 | 1.76 | 0.71 – 4.37 | 0.226 |
| NP *Pasteurella* spp. | Intercept | -3.33 | 1.02 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 2.21 | 1.03 | 9.08 | 1.21 – 67.9 | 0.032 |
| NP *B. Bronchiseptica* | Intercept | -1.35 | 0.16 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.46 | 0.30 | 1.58 | 0.87 – 2.87 | 0.130 |
| NP non-haemolytic *Streptococcus* spp. | Intercept | -1.65 | 0.49 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.46 | 0.51 | 1.58 | 0.58 – 4.27 | 0.370 |
| NP *Staphylococcus* spp. | Intercept | -1.69 | 0.49 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.50 | 0.51 | 1.64 | 0.61 – 4.44 | 0.326 |

*Model predicted disease perfectly as all cases had *Pasteurella* spp. isolated from nasopharyngeal swabs. Model convergence was achieved by modification of nasopharyngeal culture result for week 5 swab sample for pony 14.

# Table A2.19c: Results of univariable ordinary logistic regression (OLR) analyses of the risk of coughing with different explanatory variables

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = coughing* | | | | | | |
| TW $Log_{10}$ cfu/ml total bacteria | Intercept | -6.08 | 0.98 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.80 | 0.17 | 2.22 | 1.60 – 3.08 | <0.001 |
| TW $Log_{10}$ cfu/ml total *S. zooepidemicus* | Intercept | -3.49 | 0.44 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.46 | 0.10 | 1.59 | 1.32 – 1.92 | <0.001 |
| TW *S. zooepidemicus* (categorical) | Intercept | -2.35 | 0.25 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | 0.46 | 0.47 | 1.59 | 0.63 – 4.03 | 0.329 |
| | $10^5$-$10^6$ cfu/ml | 1.91 | 0.46 | 6.76 | 2.75 – 16.6 | <0.001 |
| | >$10^6$ cfu/ml | 2.27 | 0.46 | 9.70 | 3.96 – 13.8 | <0.001 |
| TW $Log_{10}$ cfu/ml *A. equuli* | Intercept | -1.77 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.15 | 0.13 | 1.17 | 0.90 – 1.52 | 0.247 |
| TW *A. equuli* (categorical) | Intercept | -1.77 | 0.18 | | | |
| | Not isolated | referent | | | | |
| | <$10^2$ cfu/ml | 0.23 | 0.48 | 1.26 | 0.49 – 3.25 | 0.638 |
| | ≥$10^2$ cfu/ml | 0.46 | 0.46 | 1.58 | 0.64 – 3.91 | 0.324 |
| TW $Log_{10}$ cfu/ml *Pasteurella* spp. | Intercept | -1.51 | 0.24 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.06 | 0.07 | 0.94 | 0.83 – 1.07 | 0.361 |
| TW *Pasteurella* spp. (categorical) | Intercept | -1.60 | 0.20 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | -0.72 | 0.51 | 0.49 | 0.18 – 1.32 | 0.158 |
| | $10^5$-$10^6$ cfu/ml | -0.05 | 0.42 | 0.95 | 0.42 – 2.14 | 0.904 |
| | >$10^6$ cfu/ml | 0.32 | 0.54 | 1.38 | 0.48 – 4.00 | 0.554 |
| TW $Log_{10}$ cfu/ml *B. bronchiseptica* | Intercept | -1.80 | 0.19 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.08 | 0.07 | 1.09 | 0.95 – 1.24 | 0.213 |
| TW *B. bronchiseptica* (categorical) | Intercept | -1.85 | 0.20 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | 0.42 | 0.50 | 1.52 | 0.58 – 4.01 | 0.398 |
| | $10^3$-$10^5$ cfu/ml | 0.90 | 0.49 | 1.46 | 0.95 – 6.40 | 0.064 |
| | >$10^5$ cfu/ml | 0.17 | 0.49 | 1.19 | 0.46 – 3.08 | 0.724 |
| TW $Log_{10}$ cfu/ml non-haemolytic *Strep.* spp. | Intercept | -0.99 | 0.25 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.24 | 0.08 | 0.78 | 0.67 – 0.91 | 0.002 |
| TW non-haemolytic *Strep.* spp. (categorical) | Intercept | -0.60 | 0.26 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | -0.96 | 0.58 | 0.14 | 0.05 – 0.44 | 0.001 |
| | $10^3$-$10^4$ cfu/ml | -1.67 | 0.50 | 0.19 | 0.07 – 0.50 | 0.001 |
| | $10^4$-$10^5$ cfu/ml | -1.88 | 0.53 | 0.15 | 0.05 – 0.43 | <0.001 |
| | >$10^5$ cfu/ml | -0.99 | 0.42 | 0.37 | 0.16 – 0.85 | 0.019 |
| Coughing 1 week previously | Intercept | -1.92 | 0.20 | | | |
| | Absent | referent | | | | |
| | Present | 1.82 | 0.37 | 6.20 | 3.01 – 12.8 | <0.001 |
| Coughing 2 weeks previously | Intercept | -1.75 | 0.20 | | | |
| | Absent | referent | | | | |
| | Present | 1.47 | 0.40 | 4.34 | 2.00 – 9.45 | <0.001 |
| Coughing 3 weeks previously | Intercept | -1.59 | 0.20 | | | |
| | Absent | referent | | | | |
| | Present | 1.08 | 0.42 | 2.94 | 1.29 – 6.67 | 0.010 |

Table A2.19c continued

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = coughing* | | | | | | |
| Sex | Intercept | -1.42 | 0.20 | | | |
| | Female | referent | | | | |
| | Male | -0.66 | 0.32 | 0.52 | 0.27 – 0.97 | 0.041 |
| Vaccine group | Intercept | -2.30 | 0.30 | | | |
| | Vaccine | referent | | | | |
| | Placebo | 1.07 | 0.37 | 2.94 | 1.43 – 6.04 | 0.003 |
| | Late intro | 0.38 | 0.51 | 1.46 | 0.54 – 3.93 | 0.455 |
| Transferrin D haplotype | Intercept | -1.25 | 0.22 | | | |
| | Absent | referent | | | | |
| | Present | -0.83 | 0.31 | 0.44 | 0.24 – 0.81 | 0.008 |
| Transferrin F2 haplotype | Intercept | -2.30 | 0.27 | | | |
| | Absent | referent | | | | |
| | Present | 1.04 | 0.33 | 2.83 | 1.47 – 5.44 | 0.002 |
| Transferrin H1 haplotype | Intercept | -1.86 | 0.17 | | | |
| | Absent | referent | | | | |
| | Present | 1.18 | 0.41 | 3.26 | 1.47 – 7.26 | 0.004 |
| Transferrin H2 haplotype | Intercept | -1.71 | 0.17 | | | |
| | Absent | referent | | | | |
| | Present | 0.05 | 0.44 | 1.05 | 0.44 – 2.51 | 0.913 |
| Transferrin O haplotype | Intercept | -1.67 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | -0.14 | 0.34 | 0.87 | 0.45 – 1.70 | 0.686 |
| Transferrin R haplotype | Intercept | -1.61 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | -1.01 | 0.62 | 0.36 | 0.11 – 1.23 | 0.103 |
| Protease inhibitor I haplotype | Intercept | -2.05 | 0.24 | | | |
| | Absent | referent | | | | |
| | Present | 0.69 | 0.32 | 1.98 | 1.07 – 3.68 | 0.030 |
| Protease inhibitor L haplotype | Intercept | -0.56 | 0.36 | | | |
| | Absent | referent | | | | |
| | Present | -1.35 | 0.40 | 0.26 | 0.12 – 0.57 | 0.001 |
| Protease inhibitor L2 haplotype | Intercept | -1.74 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | 0.76 | 0.70 | 2.14 | 0.55 – 8.35 | 0.275 |
| Protease inhibitor R haplotype | Intercept | -1.74 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | 0.76 | 0.70 | 2.14 | 0.55 – 8.35 | 0.275 |
| Protease inhibitor S haplotype | Intercept | -1.58 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | -0.96 | 0.54 | 0.38 | 0.13 – 1.11 | 0.077 |
| NP *S. zooepidemicus* | Intercept | -3.66 | 1.01 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 2.09 | 1.03 | 8.10 | 1.09 – 60.4 | 0.041 |
| NP *Pasteurella* spp. | Intercept | -3.33 | 1.02 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 1.71 | 1.03 | 5.55 | 0.74 – 41.8 | 0.096 |
| NP *B. Bronchiseptica* | Intercept | -1.80 | 0.18 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.38 | 0.35 | 1.46 | 0.74 – 2.90 | 0.277 |
| NP non-haemolytic *Streptococcus* spp. | Intercept | -2.67 | 0.73 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 1.04 | 0.75 | 2.83 | 0.65 – 12.3 | 0.165 |
| NP *Staphylococcus* spp. | Intercept | -2.71 | 0.73 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 1.08 | 0.75 | 2.94 | 0.68 – 12.7 | 0.149 |

*Model predicted disease perfectly as all cases had *Pasteurella* spp. isolated from nasopharyngeal swabs. Model convergence was achieved by modification of nasopharyngeal culture result for week 5 swab sample for pony 14.

# Table A2.19d: Results of univariable ordinary logistic regression (OLR) analyses of the risk of abnormal breathing/dyspnoea with different explanatory variables

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = abnormal breathing/dyspnoea* | | | | | | |
| TW $Log_{10}$ cfu/ml total bacteria | Intercept | -.3.39 | 0.66 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.46 | 0.12 | 1.59 | 1.26 – 2.01 | <0.001 |
| TW $Log_{10}$ cfu/ml total *S. zooepidemicus* | Intercept | -2.40 | 0.33 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.40 | 0.08 | 1.49 | 1.28 – 1.74 | <0.001 |
| TW *S. zooepidemicus* (categorical) | Intercept | -1.33 | 0.17 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | 0.31 | 0.36 | 1.36 | 0.68 – 2.73 | 0.386 |
| | $10^5$-$10^6$ cfu/ml | 1.62 | 0.42 | 5.05 | 2.23 – 11.5 | <0.001 |
| | >$10^6$ cfu/ml | 1.71 | 0.17 | 5.51 | 2.39 – 12.7 | <0.001 |
| TW $Log_{10}$ cfu/ml *A. equuli* | Intercept | -0.90 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.07 | 0.13 | 0.94 | 0.73 – 1.20 | 0.600 |
| TW *A. equuli* (categorical) | Intercept | -0.89 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | <$10^2$ cfu/ml | -0.13 | 0.41 | 0.88 | 0.39 – 1.97 | 0.747 |
| | ≥$10^2$ cfu/ml | -0.25 | 0.43 | 0.78 | 0.34 – 1.81 | 0.559 |
| TW $Log_{10}$ cfu/ml *Pasteurella* spp. | Intercept | -1.05 | 0.21 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.04 | 0.05 | 1.04 | 0.94 – 1.16 | 0.438 |
| TW *Pasteurella* spp. (categorical) | Intercept | -1.09 | 0.17 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | 0.09 | 0.35 | 1.09 | 0.55 – 2.15 | 0.805 |
| | $10^5$-$10^6$ cfu/ml | 0.34 | 0.33 | 1.41 | 0.73 – 2.71 | 0.303 |
| | >$10^6$ cfu/ml | 1.00 | 0.45 | 2.73 | 1.13 – 6.61 | 0.026 |
| TW $Log_{10}$ cfu/ml *B. bronchiseptica* | Intercept | -0.90 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.03 | 0.06 | 0.97 | 0.87 – 1.10 | 0.671 |
| TW *B. bronchiseptica* (categorical) | Intercept | -0.89 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | -0.17 | 0.44 | 0.85 | 0.36 – 1.99 | 0.705 |
| | $10^3$-$10^5$ cfu/ml | 0.14 | 0.45 | 1.15 | 0.47 – 2.79 | 0.762 |
| | >$10^5$ cfu/ml | -0.28 | 0.41 | 0.76 | 0.34 – 1.69 | 0.495 |
| TW $Log_{10}$ cfu/ml non-haemolytic *Strep.* spp. | Intercept | -0.41 | 0.22 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.17 | 0.06 | 0.84 | 0.75 – 0.95 | 0.007 |
| TW non-haemolytic *Strep.* spp. (categorical) | Intercept | -0.03 | 0.25 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | -1.17 | 0.40 | 0.31 | 0.14 – 0.69 | 0.004 |
| | $10^3$-$10^4$ cfu/ml | -1.44 | 0.41 | 0.34 | 0.11 – 0.53 | <0.001 |
| | $10^4$-$10^5$ cfu/ml | -1.36 | 0.40 | 0.26 | 0.12 – 0.56 | 0.001 |
| | >$10^5$ cfu/ml | -0.85 | 0.37 | 0.43 | 0.21 – 0.88 | 0.021 |
| Abnormal breathing 1 week previously | Intercept | -1.19 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 1.46 | 0.29 | 4.32 | 2.47 – 7.55 | <0.001 |
| Abnormal breathing 2 weeks previously | Intercept | -1.15 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 1.67 | 0.31 | 5.34 | 2.92 – 9.76 | <0.001 |
| Abnormal breathing 3 weeks previously | Intercept | -0.84 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 1.23 | 0.32 | 3.43 | 1.81 – 6.48 | <0.001 |

Table A2.19d continued

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = abnormal breathing/dyspnoea* | | | | | | |
| Sex | Intercept | -0.61 | 0.16 | | | |
| | Female | referent | | | | |
| | Male | -0.77 | 0.26 | 0.46 | 0.28 – 0.77 | 0.003 |
| Vaccine group | Intercept | -1.02 | 0.20 | | | |
| | Vaccine | referent | | | | |
| | Placebo | -0.08 | 0.28 | 0.92 | 0.53 – 1.60 | 0.778 |
| | Late intro | 0.54 | 0.34 | 1.71 | 0.87 – 3.34 | 0.114 |
| Transferrin D haplotype | Intercept | -0.22 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | -1.32 | 0.26 | 0.27 | 0.16 – 0.44 | <0.001 |
| Transferrin F2 haplotype | Intercept | -1.93 | 0.23 | | | |
| | Absent | referent | | | | |
| | Present | 1.69 | 0.28 | 5.42 | 3.10 – 9.47 | <0.001 |
| Transferrin H1 haplotype | Intercept | -1.00 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | 0.44 | 0.39 | 1.55 | 0.73 – 3.30 | 0.255 |
| Transferrin H2 haplotype | Intercept | -0.93 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | -0.17 | 0.37 | 0.84 | 0.41 – 1.75 | 0.644 |
| Transferrin O haplotype | Intercept | -0.83 | 0.15 | | | |
| | Absent | referent | | | | |
| | Present | -0.43 | 0.28 | 0.65 | 0.37 – 1.14 | 0.131 |
| Transferrin R haplotype | Intercept | -0.96 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | 0.09 | 0.36 | 1.10 | 0.55 – 2.21 | 0.793 |
| Protease inhibitor I haplotype | Intercept | -1.07 | 0.17 | | | |
| | Absent | referent | | | | |
| | Present | 0.26 | 0.25 | 1.29 | 0.79 – 2.11 | 0.304 |
| Protease inhibitor L haplotype | Intercept | 0.31 | 0.35 | | | |
| | Absent | referent | | | | |
| | Present | -1.43 | 0.38 | 0.24 | 0.11 – 0.50 | <0.001 |
| Protease inhibitor L2 haplotype | Intercept | -1.00 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | 1.18 | 0.62 | 3.25 | 0.97 – 10.9 | 0.057 |
| Protease inhibitor R haplotype | Intercept | -0.42 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | -1.39 | 1.06 | 0.25 | 0.03 – 1.98 | 0.189 |
| Protease inhibitor S haplotype | Intercept | -1.06 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | 0.58 | 0.31 | 1.78 | 0.97 – 3.28 | 0.064 |
| NP *S. zooepidemicus* | Intercept | -3.66 | 1.01 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 2.89 | 1.02 | 18.0 | 2.43 – 132.9 | 0.005 |
| NP *Pasteurella* spp. | Intercept | -3.33 | 1.02 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 2.50 | 1.03 | 12.2 | 1.63 – 91.1 | 0.015 |
| NP *B. Bronchiseptica* | Intercept | -0.95 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.01 | 0.30 | 0.99 | 0.55 – 1.78 | 0.979 |
| NP non-haemolytic *Streptococcus* spp. | Intercept | -1.23 | 0.43 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.31 | 0.45 | 1.36 | 0.57 – 3.29 | 0.489 |
| NP *Staphylococcus* spp. | Intercept | -1.27 | 0.43 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.36 | 0.45 | 1.43 | 0.59 – 3.43 | 0.425 |

*Model predicted disease perfectly as all cases had *Pasteurella* spp. isolated from nasopharyngeal swabs. Model convergence was achieved by modification of nasopharyngeal culture result for week 5 swab sample for pony 14.

# Table A2.19e: Results of univariable ordinary logistic regression (OLR) analyses of the risk of SMLN enlargement with different explanatory variables

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = SMLN enlargement* | | | | | | |
| TW $Log_{10}$ cfu/ml total bacteria | Intercept | -3.40 | 0.65 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.48 | 0.12 | 1.62 | 1.29 – 2.04 | <0.001 |
| TW $Log_{10}$ cfu/ml total *S. zooepidemicus* | Intercept | -1.93 | 0.30 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.31 | 0.07 | 1.36 | 1.18 – 1.57 | <0.001 |
| TW *S. zooepidemicus* (categorical) | Intercept | -1.22 | 0.17 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | 0.64 | 0.33 | 1.89 | 0.99 – 3.62 | 0.054 |
| | $10^5$-$10^6$ cfu/ml | 1.08 | 0.41 | 2.93 | 1.30 – 6.60 | 0.009 |
| | >$10^6$ cfu/ml | 1.59 | 0.43 | 4.92 | 2.14 – 11.3 | <0.001 |
| TW $Log_{10}$ cfu/ml *A. equuli* | Intercept | -0.76 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.19 | 0.13 | 0.83 | 0.64 – 1.08 | 0.168 |
| TW *A. equuli* (categorical) | Intercept | -0.74 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | <$10^2$ cfu/ml | -0.29 | 0.41 | 0.75 | 0.34 – 1.68 | 0.488 |
| | ≥$10^2$ cfu/ml | -0.77 | 0.47 | 0.46 | 0.18 – 1.17 | 0.103 |
| TW $Log_{10}$ cfu/ml *Pasteurella* spp. | Intercept | -0.96 | 0.20 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.04 | 0.05 | 1.04 | 0.94 – 1.15 | 0.447 |
| TW *Pasteurella* spp. (categorical) | Intercept | -1.03 | 0.17 | | | |
| | <$10^4$ cfu/ml | referent | | | | |
| | $10^4$-$10^5$ cfu/ml | 0.12 | 0.34 | 1.12 | 0.58 – 2.19 | 0.733 |
| | $10^5$-$10^6$ cfu/ml | 0.52 | 0.32 | 1.69 | 0.89 – 3.18 | 0.107 |
| | >$10^6$ cfu/ml | 0.95 | 0.45 | 2.57 | 1.06 – 6.23 | 0.036 |
| TW $Log_{10}$ cfu/ml *B. bronchiseptica* | Intercept | -0.93 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.07 | 0.06 | 1.07 | 0.96 – 1.20 | 0.215 |
| TW *B. bronchiseptica* (categorical) | Intercept | -0.96 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | 0.36 | 0.40 | 1.43 | 0.65 – 3.17 | 0.373 |
| | $10^3$-$10^5$ cfu/ml | 0.88 | 0.43 | 2.41 | 1.04 – 5.56 | 0.040 |
| | >$10^5$ cfu/ml | 0.06 | 0.39 | 1.06 | 0.50 – 2.27 | 0.877 |
| TW $Log_{10}$ cfu/ml non-haemolytic *Strep.* spp. | Intercept | -0.23 | 0.22 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.20 | 0.06 | 0.82 | 0.73 – 0.93 | 0.001 |
| TW non-haemolytic *Strep.* spp. (categorical) | Intercept | 0.03 | 0.25 | | | |
| | Not isolated | referent | | | | |
| | <$10^3$ cfu/ml | -0.70 | 0.38 | 0.50 | 0.24 – 1.04 | 0.064 |
| | $10^3$-$10^4$ cfu/ml | -1.98 | 0.45 | 0.14 | 0.06 – 0.37 | <0.001 |
| | $10^4$-$10^5$ cfu/ml | -0.99 | 0.37 | 0.37 | 0.18 – 0.77 | 0.008 |
| | >$10^5$ cfu/ml | -1.07 | 0.38 | 0.34 | 0.16 – 0.72 | 0.004 |
| SMLN enlargement 1 week previously | Intercept | -1.52 | 0.19 | | | |
| | Absent | referent | | | | |
| | Present | 2.31 | 0.31 | 10.1 | 5.48 – 18.6 | <0.001 |
| SMLN enlargement 2 weeks previously | Intercept | -1.09 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 1.59 | 0.31 | 4.92 | 2.70 – 8.98 | <0.001 |
| SMLN enlargement 3 weeks previously | Intercept | -0.94 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 1.26 | 0.33 | 3.52 | 1.86 – 6.66 | <0.001 |

Table A2.19e continued

| Explanatory variable | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| *Outcome = SMLN enlargement* | | | | | | |
| Sex | Intercept | -0.83 | 0.17 | | | |
| | Female | referent | | | | |
| | Male | -0.05 | 0.25 | 0.95 | 0.59 – 1.54 | 0.833 |
| Vaccine group | Intercept | -1.27 | 0.21 | | | |
| | Vaccine | referent | | | | |
| | Placebo | 0.21 | 0.29 | 1.23 | 0.70 – 2.17 | 0.471 |
| | Late intro | 1.60 | 0.34 | 4.94 | 2.51 – 9.71 | <0.001 |
| Transferrin D haplotype | Intercept | -0.67 | 0.19 | | | |
| | Absent | referent | | | | |
| | Present | -0.31 | 0.25 | 0.73 | 0.45 – 1.19 | 0.211 |
| Transferrin F2 haplotype | Intercept | -1.07 | 0.18 | | | |
| | Absent | referent | | | | |
| | Present | 0.43 | 0.25 | 1.54 | 0.95 – 2.49 | 0.081 |
| Transferrin I11 haplotype | Intercept | -0.76 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | -1.22 | 0.55 | 0.30 | 0.10 – 0.87 | 0.026 |
| Transferrin I12 haplotype | Intercept | -0.93 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | 0.46 | 0.34 | 1.59 | 0.82 – 3.08 | 0.164 |
| Transferrin O haplotype | Intercept | -0.85 | 0.15 | | | |
| | Absent | referent | | | | |
| | Present | -0.03 | 0.27 | 0.97 | 0.57 – 1.63 | 0.898 |
| Transferrin R haplotype | Intercept | -0.77 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | -0.73 | 0.41 | 0.48 | 0.21 – 1.08 | 0.075 |
| Protease inhibitor I haplotype | Intercept | -0.76 | 0.16 | | | |
| | Absent | referent | | | | |
| | Present | -0.22 | 0.25 | 0.80 | 0.49 – 1.31 | 0.378 |
| Protease inhibitor L haplotype | Intercept | -1.50 | 0.45 | | | |
| | Absent | referent | | | | |
| | Present | 0.71 | 0.47 | 2.03 | 0.81 – 5.10 | 0.130 |
| Protease inhibitor L2 haplotype | Intercept | -0.95 | 0.13 | | | |
| | Absent | referent | | | | |
| | Present | 2.45 | 0.79 | 11.6 | 2.46 – 54.9 | 0.002 |
| Protease inhibitor R haplotype | Intercept | -0.82 | 0.12 | | | |
| | Absent | referent | | | | |
| | Present | -1.48 | 1.06 | 0.23 | 0.03 – 1.80 | 0.161 |
| Protease inhibitor S haplotype | Intercept | -1.00 | 0.14 | | | |
| | Absent | referent | | | | |
| | Present | 0.74 | 0.31 | 2.10 | 1.16 – 3.83 | 0.015 |
| NP *S. zooepidemicus* | Intercept | -2.20 | 0.53 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 1.47 | 0.54 | 4.36 | 1.50 – 12.6 | 0.007 |
| NP *Pasteurella* spp. | Intercept | -1.10 | 0.44 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.26 | 0.45 | 1.30 | 0.53 – 3.17 | 0.563 |
| NP *B. Bronchiseptica* | Intercept | -0.91 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.21 | 0.29 | 1.24 | 0.71 – 2.17 | 0.454 |
| NP non-haemolytic *Streptococcus* spp. | Intercept | -1.23 | 0.43 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.41 | 0.45 | 1.51 | 0.63 – 3.63 | 0.359 |
| NP *Staphylococcus* spp. | Intercept | -1.27 | 0.43 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.46 | 0.45 | 1.58 | 0.66 – 3.79 | 0.306 |

**Figure A2.12a: Relationship between *S. zooepidemicus* infection and odds of nasal discharge**

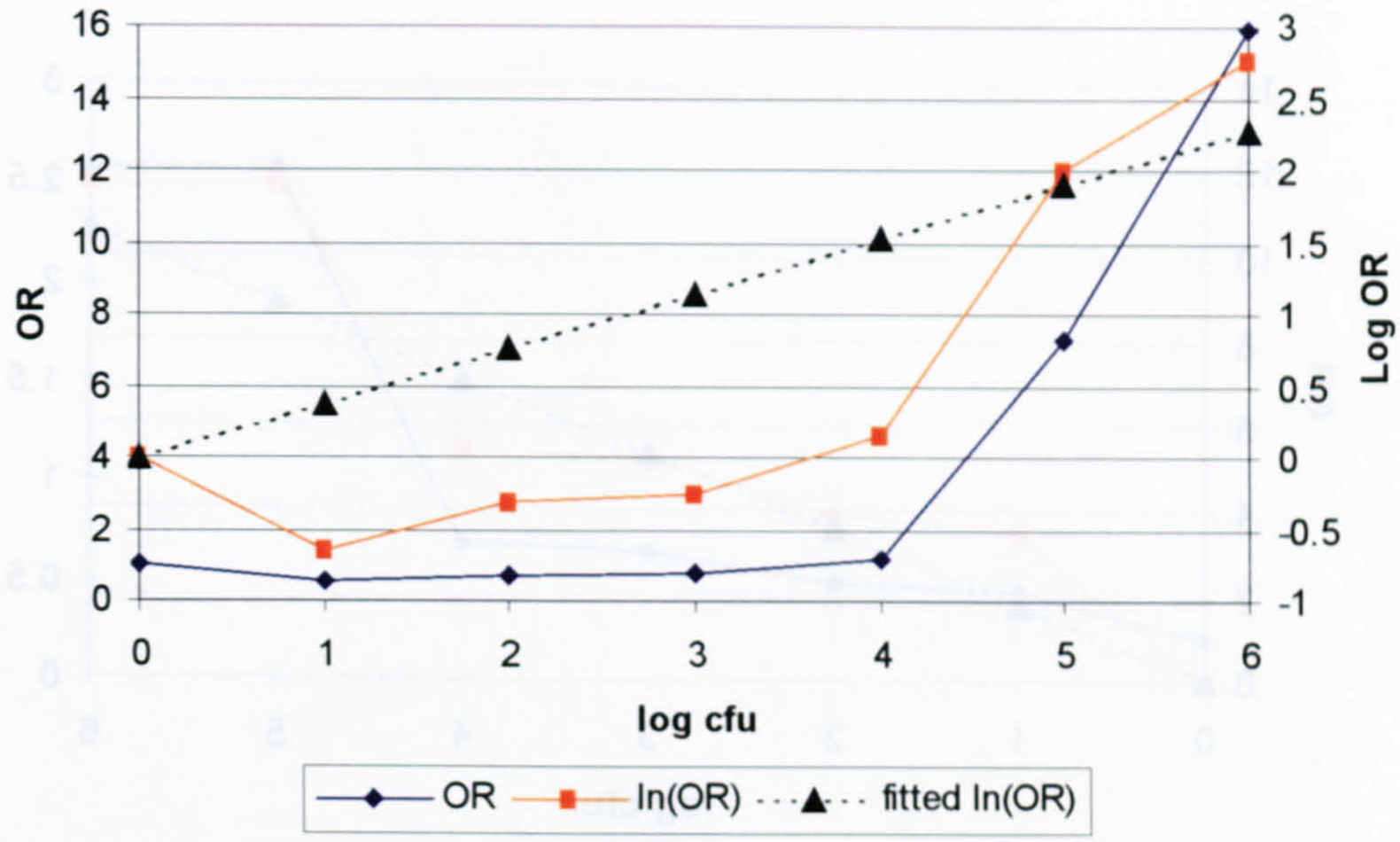



**Figure A2.12b: Relationship between *S. zooepidemicus* infection and odds of coughing**

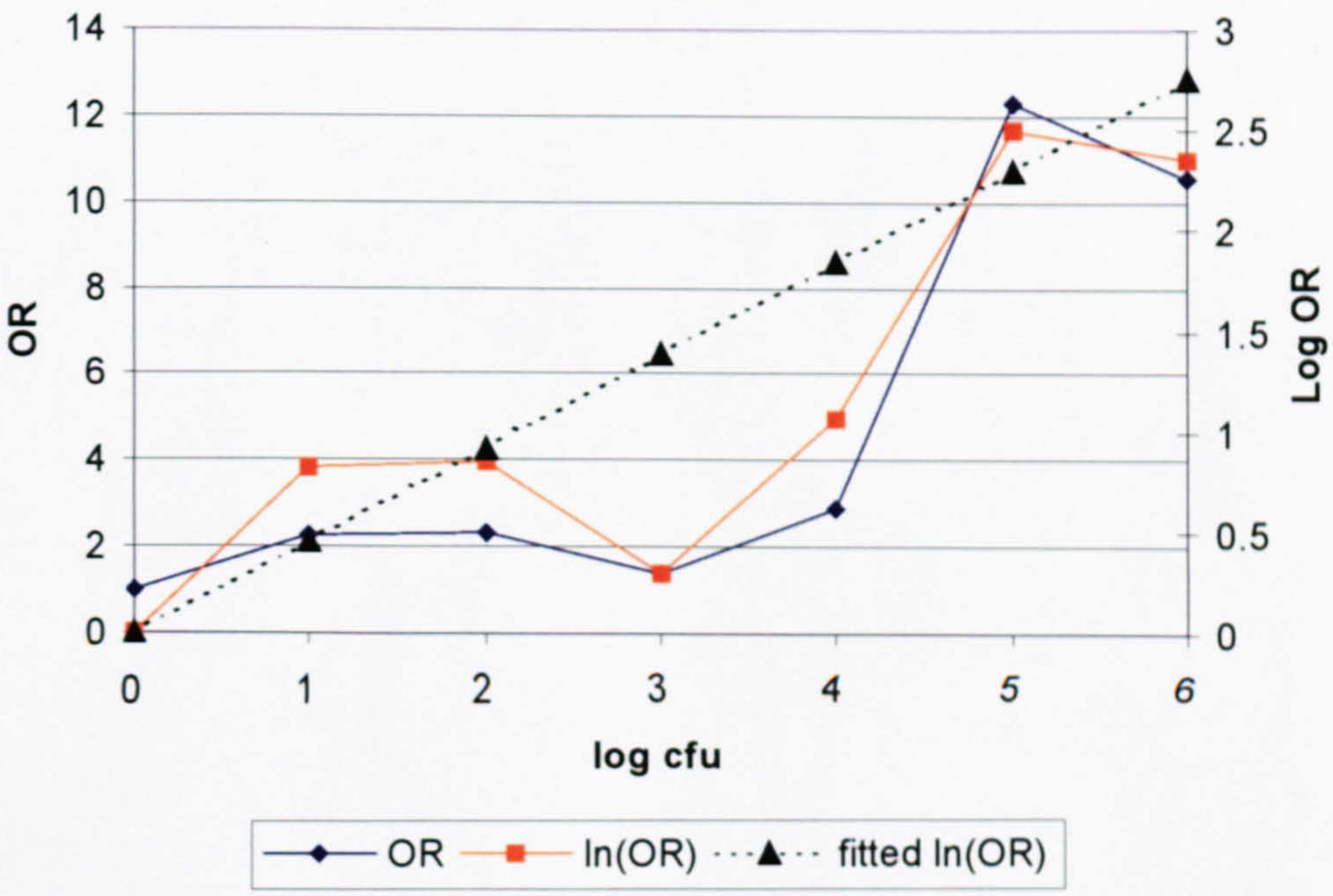


**Figure A2.12c: Relationship between *S. zooepidemicus* infection and odds of abnormal breathing/dyspnoea**

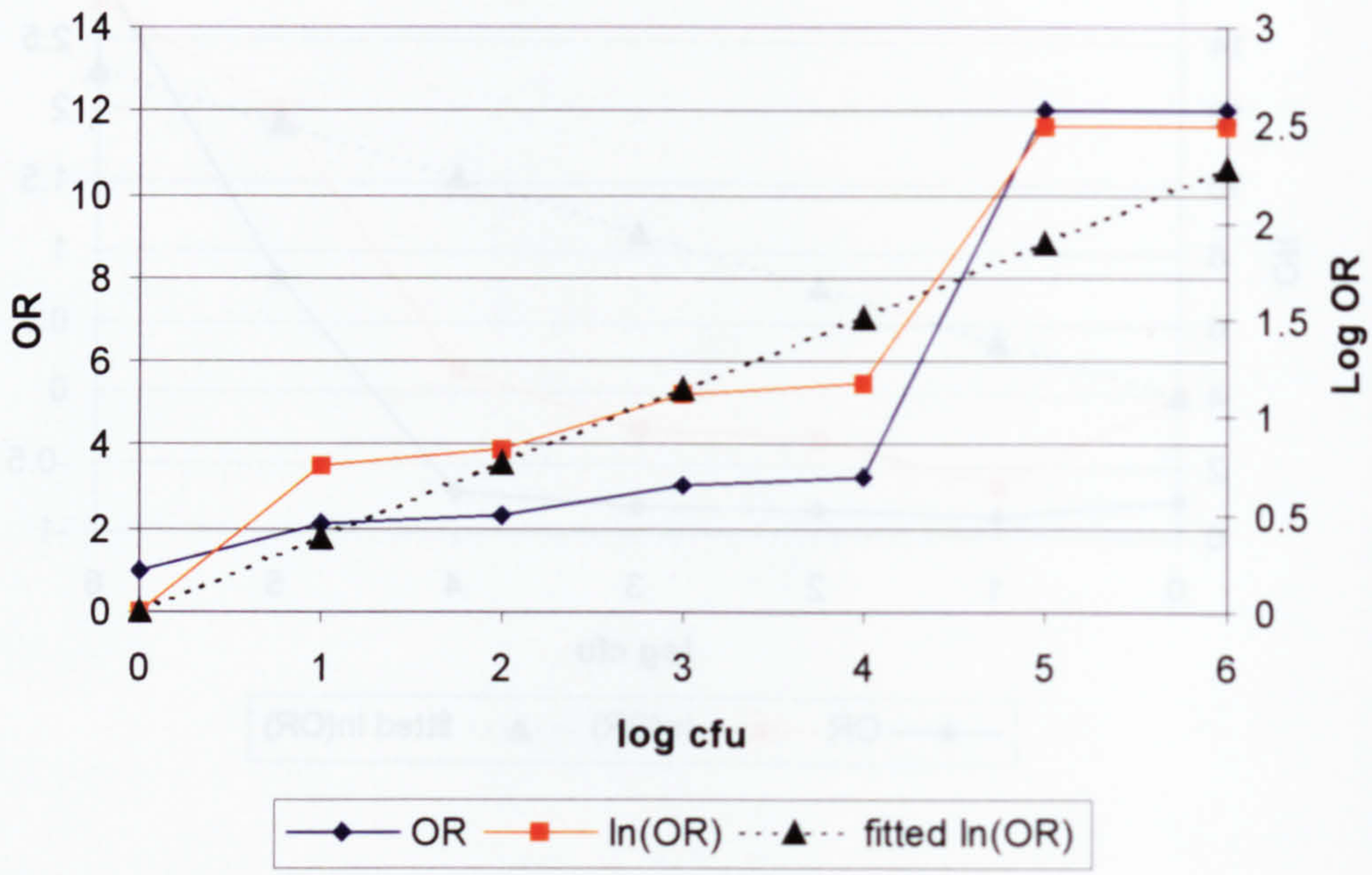


**Figure A2.13: Caterpillar plot of ranked pony level residuals (± 95% CI) estimated using an iterative generalised least squares (IGLS) algorithm for final logistic regression model for nasal discharge including random effect term**

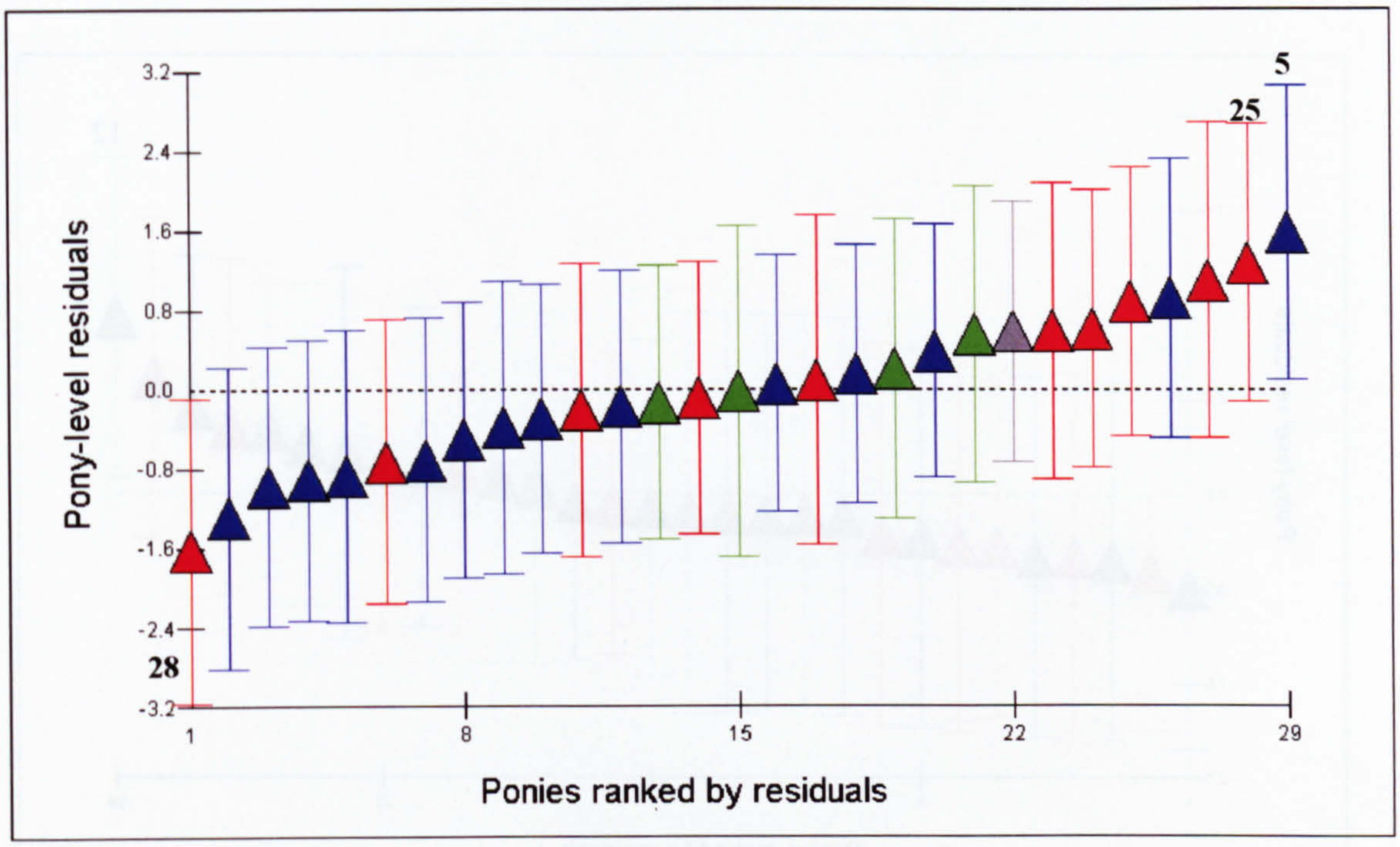
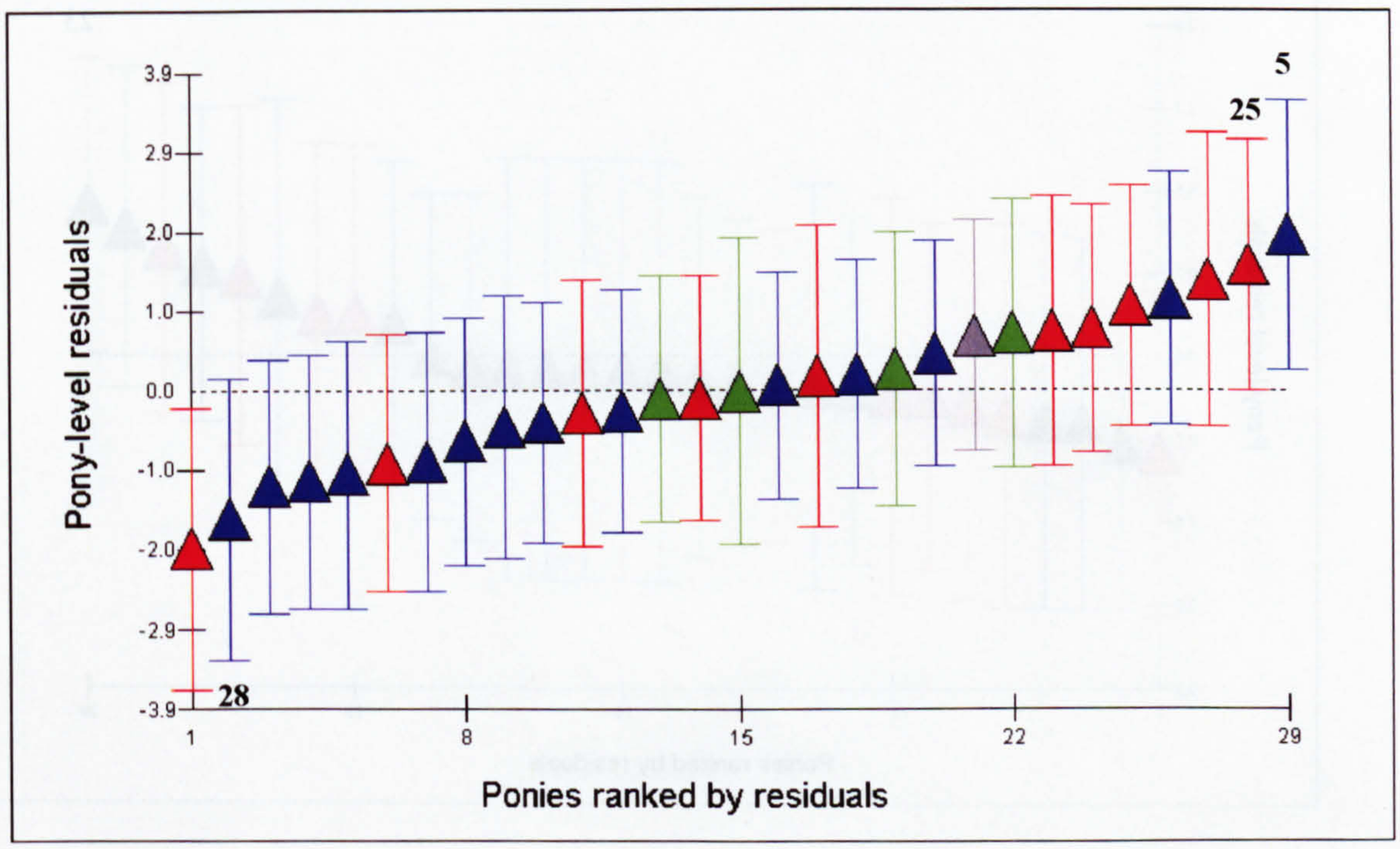



**Figure A2.14: Caterpillar plot of ranked pony level residuals (± 95% CI) estimated using a restricted iterative generalised least squares (RIGLS) algorithm for final logistic regression model for nasal discharge including random effect term**




Note: Colour scheme represents the transferrin D/F2 haplotype status of each pony light blue: transferrin D haplotype (n=14), red: transferrin F2 haplotype (n=10), light green: transferrin DF2 haplotype (n=4) & grey: other transferrin haplotype (n=1)

**Figure A2.15: Caterpillar plot of ranked pony level residuals (± 95% CI) for final logistic regression model of coughing including transferrin D haplotype and random effect terms**

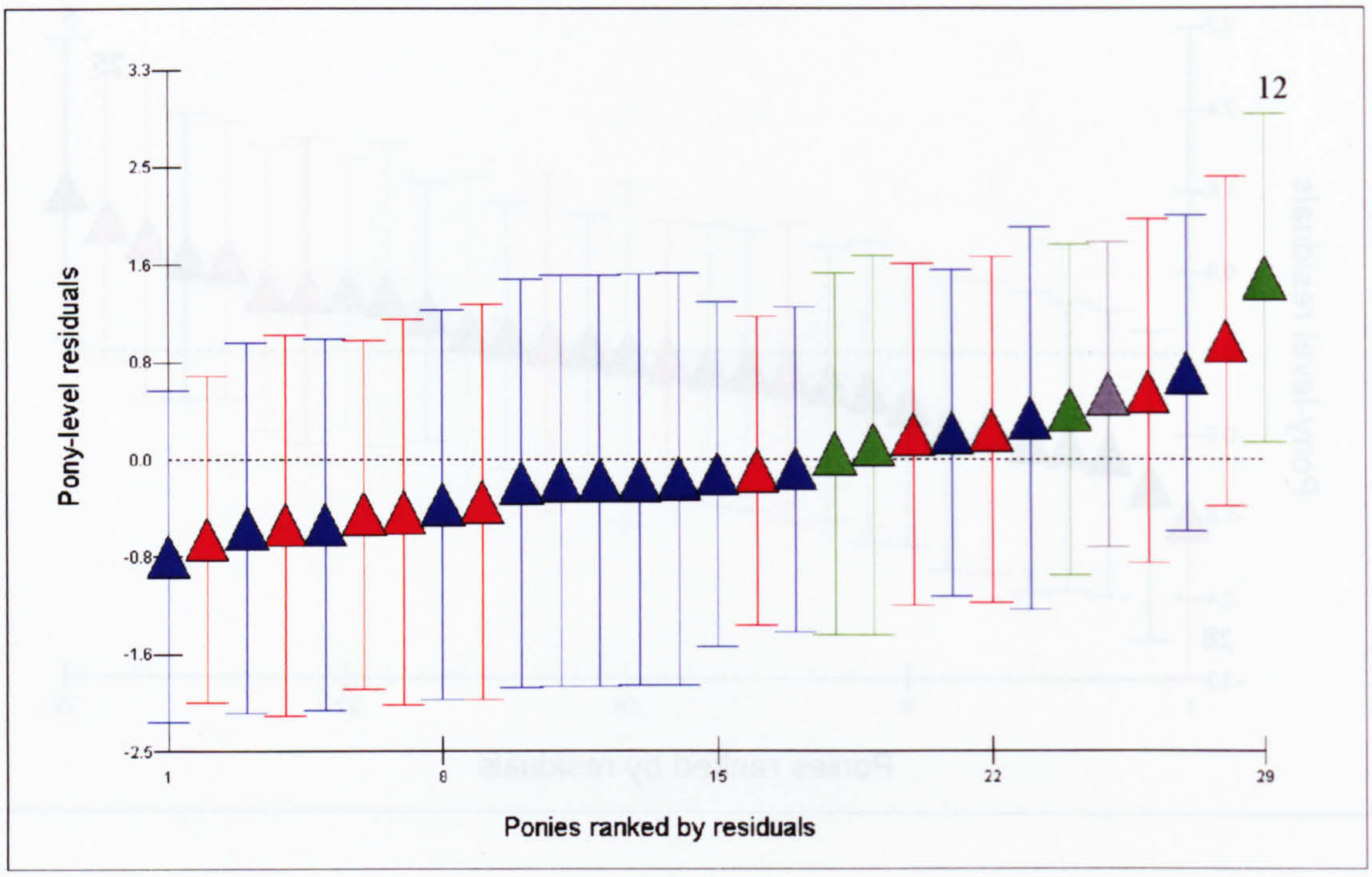
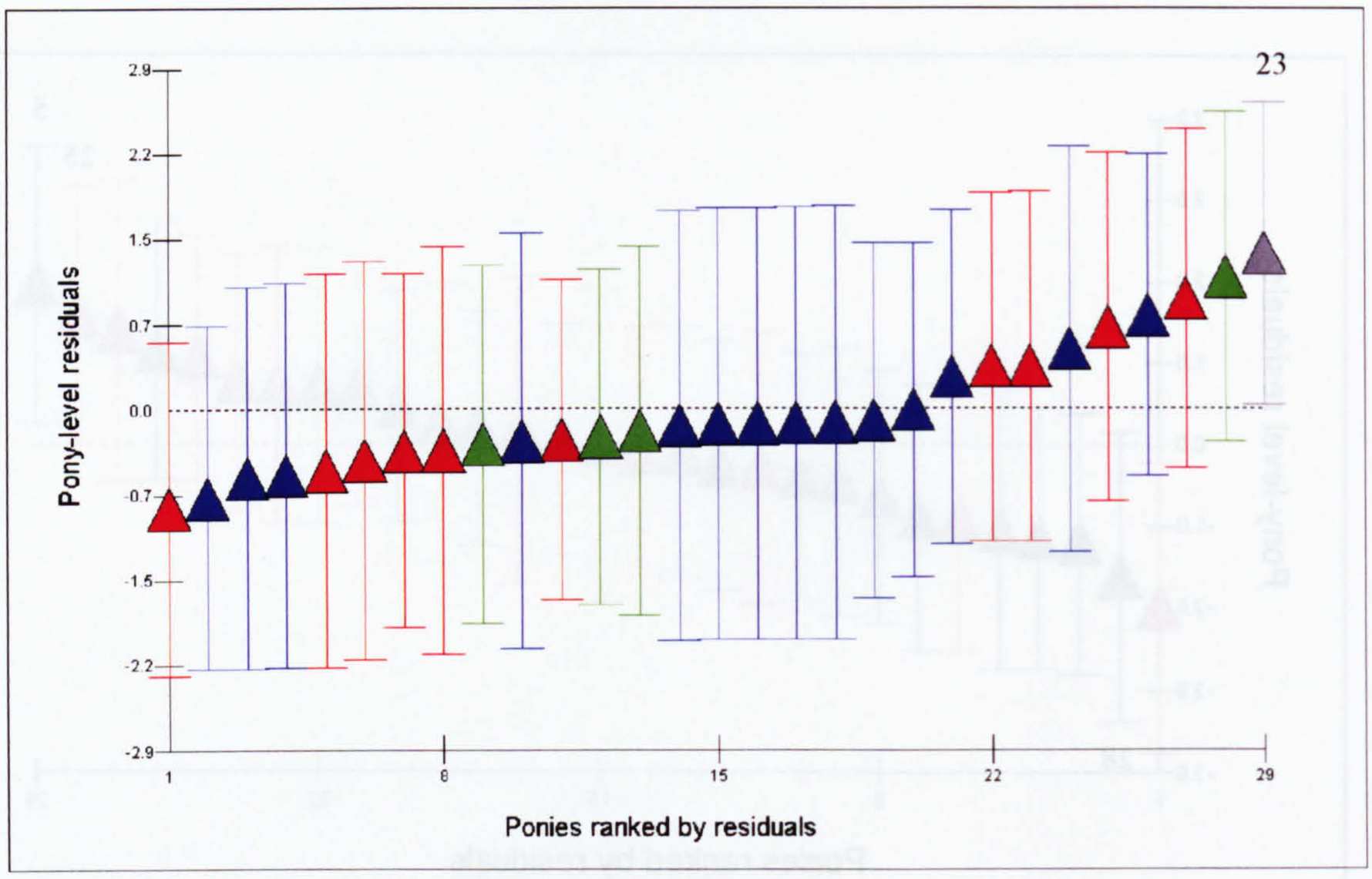



Figure A2.16: Caterpillar plot of ranked pony level residuals (± 95% CI) for final logistic regression model for coughing including transferrin F2 haplotype and random effect terms




Note: Colour scheme represents the transferrin D/F2 haplotype status of each pony light blue: transferrin D haplotype (n=14), red: transferrin F2 haplotype (n=10), light green: transferrin DF2 haplotype (n=4) & grey: other transferrin haplotype (n=1)

**Figure A2.17: Caterpillar plot of ranked pony level residuals (± 95% CI) for final logistic regression model for abnormal breathing/dyspnoea including transferrin D haplotype and random effect terms**

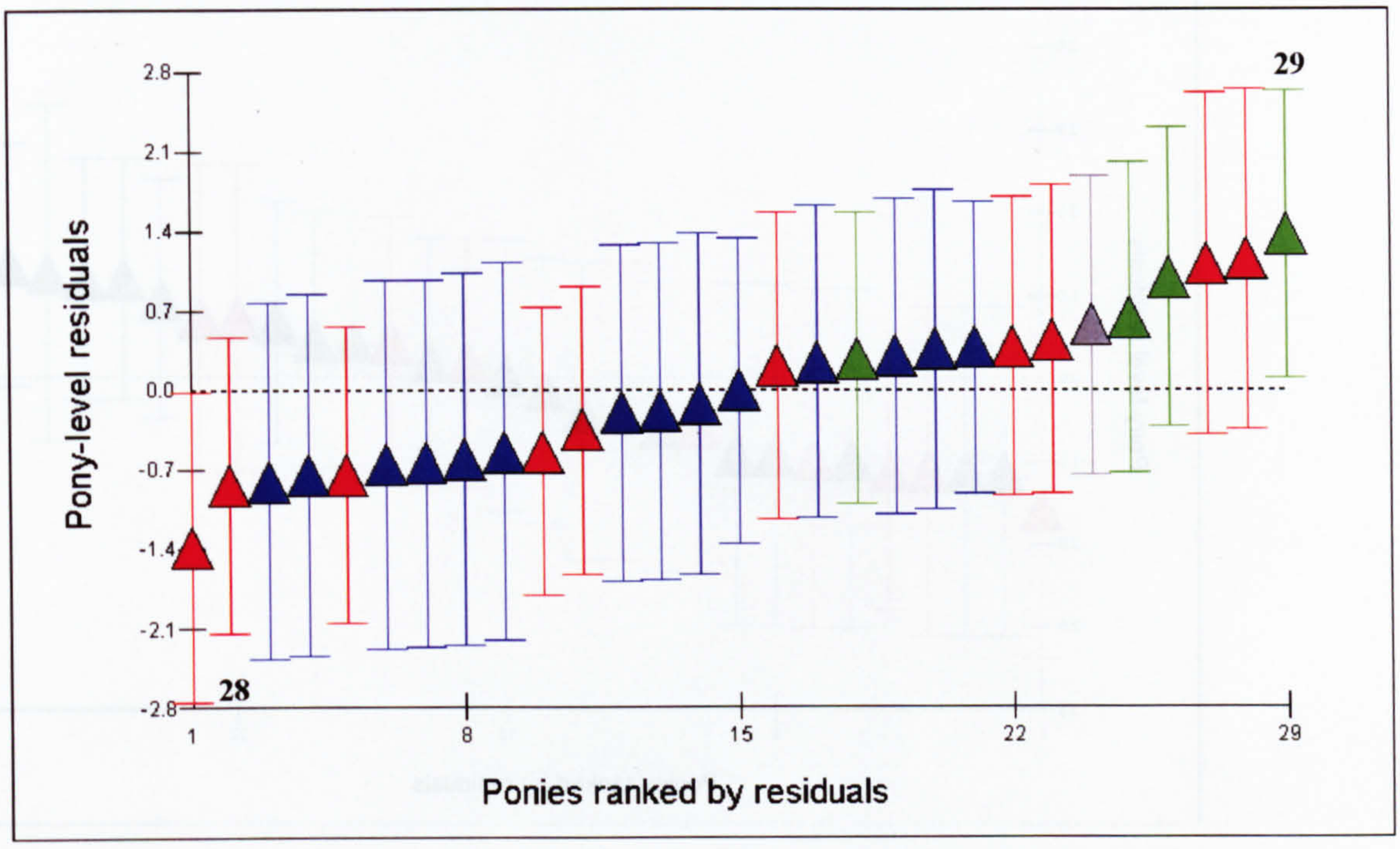
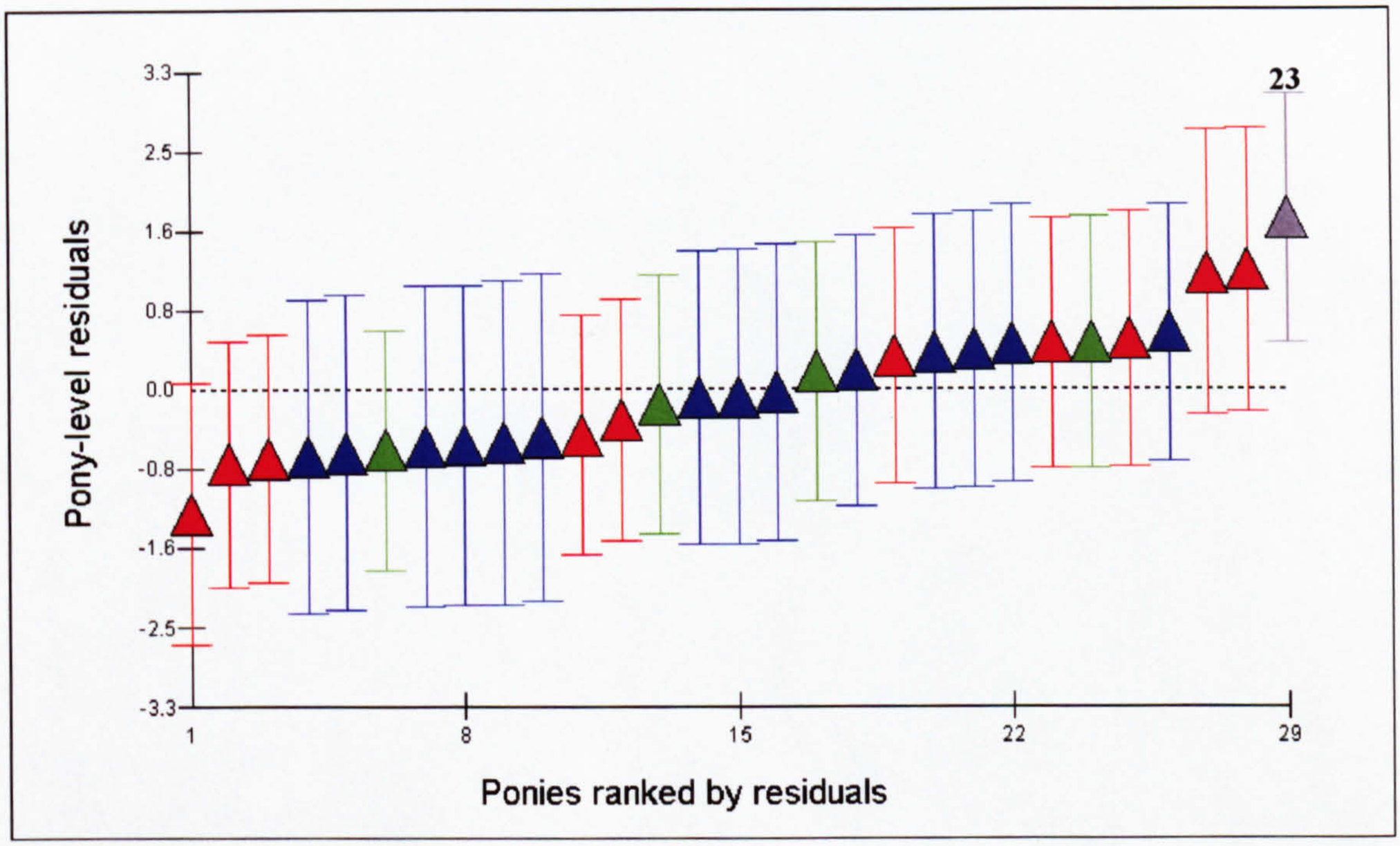
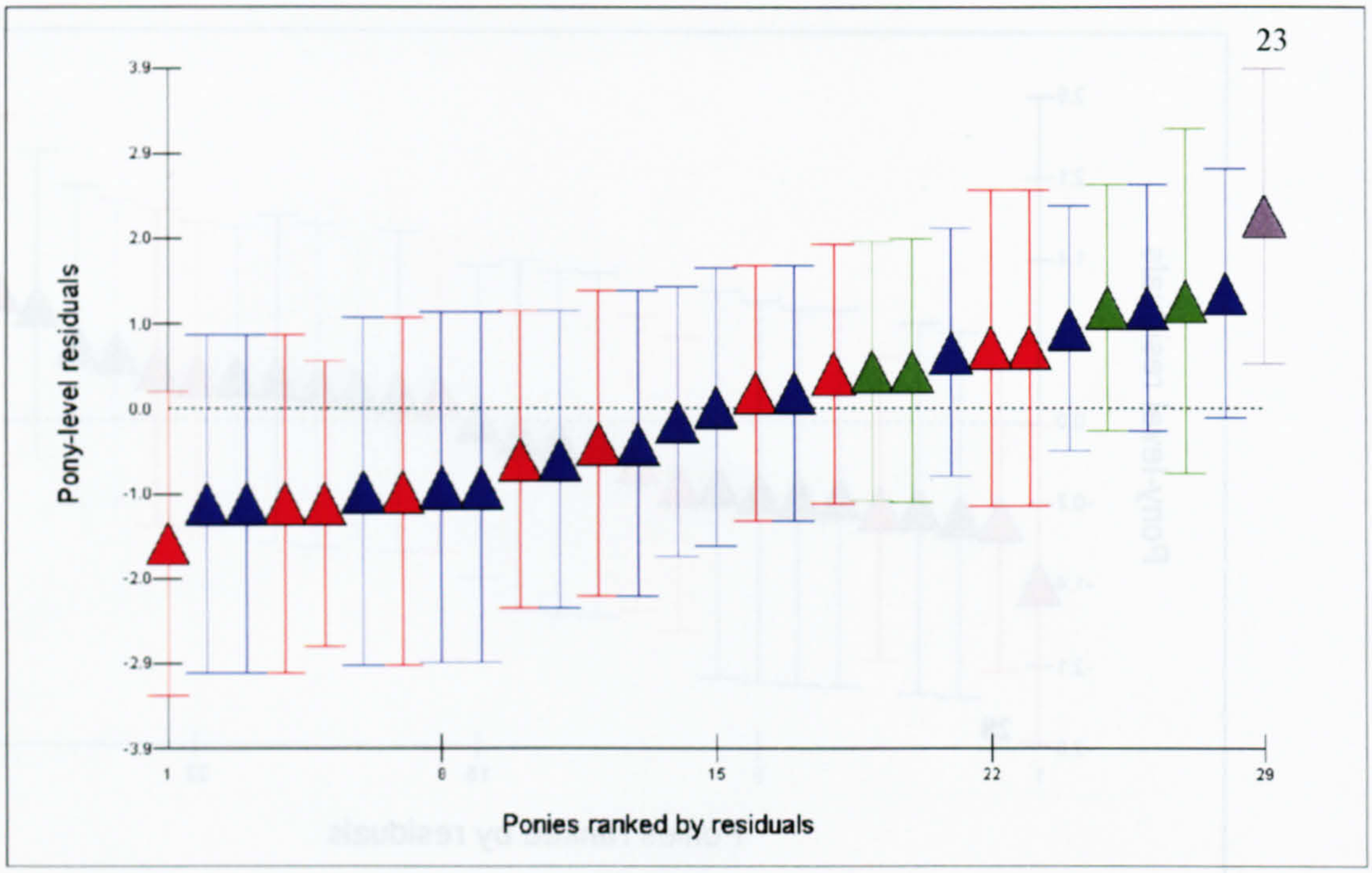



**Figure A2.18: Caterpillar plot of ranked pony level residuals (± 95% CI) for final logistic regression model for abnormal breathing/dyspnoea including transferrin F2 haplotype and random effect terms**




Note: Colour scheme represents the transferrin D/F2 haplotype status of each pony light blue: transferrin D haplotype (n=14), red: transferrin F2 haplotype (n=10), light green: transferrin DF2 haplotype (n=4) & grey: other transferrin haplotype (n=1)

**Figure A2.19: Caterpillar plot of ranked pony level residuals (± 95% CI) for final logistic regression model for SMLN enlargement and random effect term**




Note: Colour scheme represents the transferrin D/F2 haplotype status of each pony light blue: transferrin D haplotype (n=14), red: transferrin F2 haplotype (n=10), light green: transferrin DF2 haplotype (n=4) & grey: other transferrin haplotype (n=1)

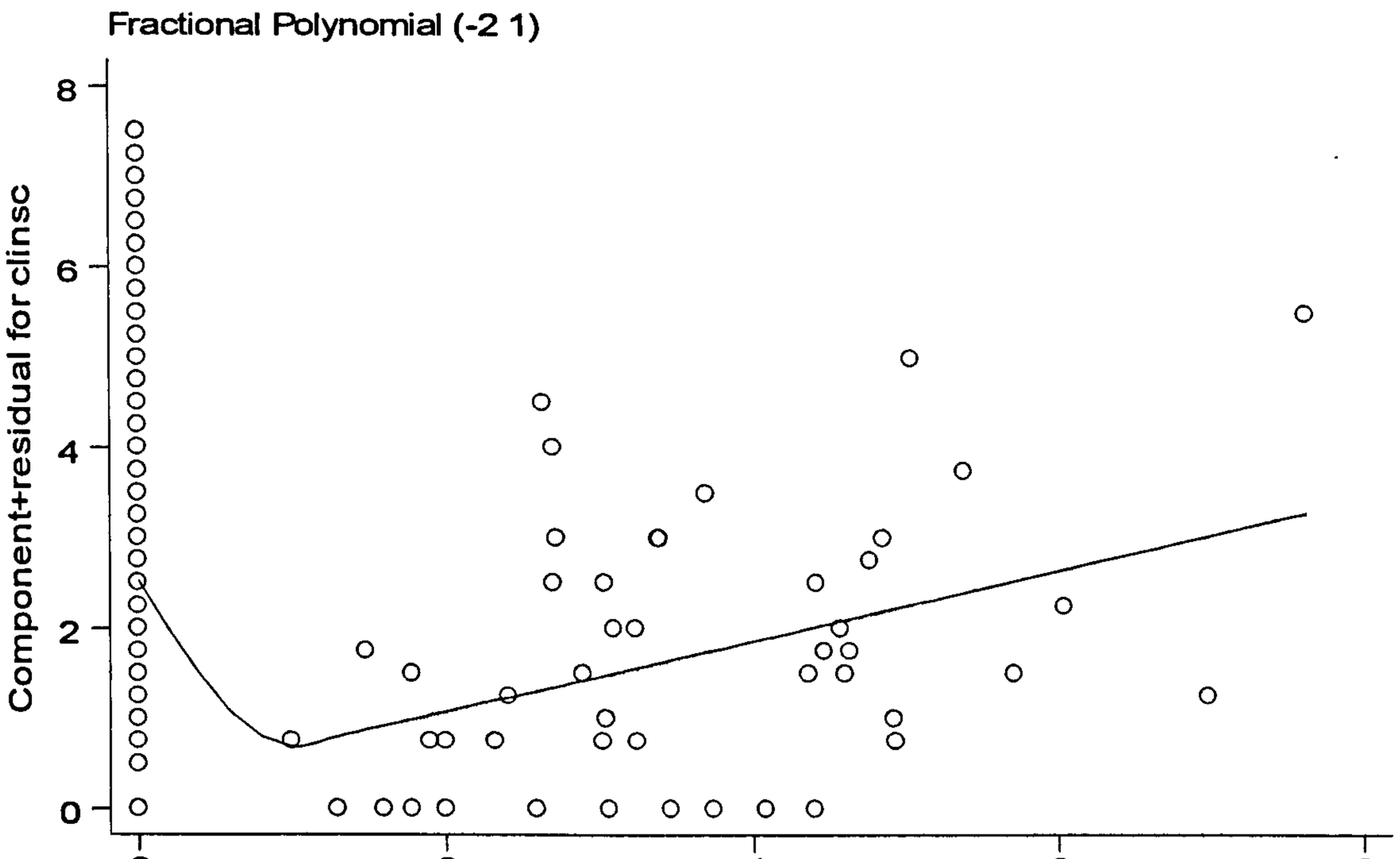
# APPENDIX 3

## Chapter 12: Tables & figures

## Table A3.1: Results and comparison of univariable linear and best fitting polynomial regressions of clinical and airway inflammation parameter scores with tracheal wash bacterial count data for the 5 most prevalent *S. zooepidemicus* types

| Outcome var. *Regression type* | Explanatory Variable | Regression coefficient | Intercept | $R^2$ value (%) | P-value | Deviance benefit | P-value ($\chi^2$, 3 df) |
|---|---|---|---|---|---|---|---|
| **Clinical score** | (n=295) | | | | | | |
| *Linear* | A1 HV1 $\log_{10}$ cfu/ml | -0.13 | 2.47 | 1.0 | 0.088 | | |
| *Polynomial* | [A1 HV1 $\log_{10}$ cfu/ml]$^{-2}$ | 0.0002 | 0.30 | 4.1 | 0.002 | | |
| | [A1 HV1 $\log_{10}$ cfu/ml]$^{1}$ | 0.39 | | | 0.036 | 9.52 | 0.023 |
| *Linear* | A1 HVu $\log_{10}$ cfu/ml | 0.28 | 2.26 | 4.3 | <0.001 | | |
| *Polynomial* | [A1 HVu $\log_{10}$ cfu/ml]$^{-0.5}$ | 0.97 | 0.57 | 4.9 | 0.013 | | |
| | $\log_n$ [A1 HVu $\log_{10}$ cfu/ml]$^{1}$ | 1.74 | | | 0.006 | 1.99 | 0.574 |
| *Linear* | A1 HV3 $\log_{10}$ cfu/ml | 0.08 | 2.37 | 0.3 | 0.872 | | |
| *Polynomial* | [A1 HV3 $\log_{10}$ cfu/ml]$^{1}$ | -1.27 | 2.50 | 4.0 | 0.002 | | |
| | [A1 HV3 $\log_{10}$ cfu/ml]$^{1}$ × $\log_n$ [x] | 0.84 | | | 0.001 | 11.11 | 0.011 |
| *Linear* | C1 HV3 $\log_{10}$ cfu/ml | 0.25 | 2.30 | 2.9 | 0.004 | | |
| *Polynomial* | $\log_n$ [C1 HV3 $\log_{10}$ cfu/ml]$^{1}$ | 1.61 | 0.006 | 4.5 | 0.001 | | |
| | ($\log_n$ [C1 HV3 $\log_{10}$ cfu/ml])$^{2}$ | 0.46 | | | 0.003 | 5.01 | 0.171 |
| *Linear* | A1 HV4 $\log_{10}$ cfu/ml | 0.27 | 2.32 | 2.5 | 0.006 | | |
| *Polynomial* | [A1 HV4 $\log_{10}$ cfu/ml]$^{-0.5}$ | -4.13 | 2.75 | 5.9 | 0.001 | | |
| | [A1 HV4 $\log_{10}$ cfu/ml]$^{1}$ | 1.99 | | | <0.001 | 10.55 | 0.014 |
| **CDNS score** | (n=295) | | | | | | |
| *Linear* | A1 HV1 $\log_{10}$ cfu/ml | -0.09 | 1.56 | 0.6 | 0.188 | | |
| *Polynomial* | [A1 HV1 $\log_{10}$ cfu/ml]$^{-2}$ | 0.0001 | 0.44 | 4.9 | <0.001 | | |
| | [A1 HV1 $\log_{10}$ cfu/ml]$^{3}$ | 0.008 | | | 0.004 | 13.13 | 0.004 |
| *Linear* | A1 HVu $\log_{10}$ cfu/ml | 0.23 | 1.41 | 4.1 | <0.001 | | |
| *Polynomial* | [A1 HVu $\log_{10}$ cfu/ml]$^{-2}$ | 0.0001 | 0.35 | 5.3 | 0.055 | | |
| | [A1 HVu $\log_{10}$ cfu/ml]$^{1}$ | 0.46 | | | 0.001 | 3.71 | 0.294 |
| *Linear* | A1 HV3 $\log_{10}$ cfu/ml | 0.08 | 1.49 | 0.3 | 0.321 | | |
| *Polynomial* | [A1 HV3 $\log_{10}$ cfu/ml]$^{1}$ | -1.02 | 1.60 | 3.7 | 0.004 | | |
| | [A1 HV3 $\log_{10}$ cfu/ml]$^{1}$ × $\log_n$ [x] | 0.68 | | | 0.002 | 10.19 | 0.017 |
| *Linear* | C1 HV3 $\log_{10}$ cfu/ml | 0.21 | 1.44 | 2.8 | 0.004 | | |
| *Polynomial* | [C1 HV3 $\log_{10}$ cfu/ml]$^{0.5}$ | -1.58 | 2.12 | 3.7 | 0.108 | | |
| | [C1 HV3 $\log_{10}$ cfu/ml]$^{0.5}$ × $\log_n$ [x] | 1.11 | | | 0.038 | 2.81 | 0.422 |
| *Linear* | A1 HV4 $\log_{10}$ cfu/ml | 0.23 | 1.46 | 2.7 | 0.005 | | |
| *Polynomial* | [A1 HV4 $\log_{10}$ cfu/ml]$^{1}$ | -1.14 | 1.53 | 6.5 | 0.005 | | |
| | [A1 HV4 $\log_{10}$ cfu/ml]$^{1}$ × $\log_n$ [x] | 0.81 | | | 0.001 | 11.94 | 0.008 |
| **Airway inflammation score** (n=295) | | | | | | | |
| *Linear* | A1 HV1 $\log_{10}$ cfu/ml | 0.10 | 6.17 | 0.5 | 0.222 | | |
| *Polynomial* | [A1 HV1 $\log_{10}$ cfu/ml]$^{-0.5}$ | 1.53 | 2.41 | 3.4 | 0.002 | | |
| | $\log_n$ [A1 HV1 $\log_{10}$ cfu/ml]$^{1}$ | 2.49 | | | 0.001 | 8.70 | 0.034 |
| *Linear* | A1 HVu $\log_{10}$ cfu/ml | 0.27 | 6.10 | 3.7 | 0.001 | | |
| *Polynomial* | [A1 HVu $\log_{10}$ cfu/ml]$^{-2}$ | 0.0002 | 3.36 | 4.6 | 0.022 | | |
| | [A1 HVu $\log_{10}$ cfu/ml]$^{0.5}$ | 1.90 | | | 0.002 | 2.86 | 0.414 |
| *Linear* | A1 HV3 $\log_{10}$ cfu/ml | 0.12 | 6.18 | 0.6 | 0.201 | | |
| *Polynomial* | [A1 HV3 $\log_{10}$ cfu/ml]$^{0.5}$ | -2.53 | 6.55 | 4.1 | 0.029 | | |
| | [A1 HV3 $\log_{10}$ cfu/ml]$^{1}$ | 1.20 | | | 0.017 | 4.82 | 0.185 |
| *Linear* | C1 HV3 $\log_{10}$ cfu/ml | 0.13 | 6.18 | 0.7 | 0.153 | | |
| *Polynomial* | $\log_n$ [C1 HV3 $\log_{10}$ cfu/ml]$^{1}$ | 1.56 | 3.33 | 3.1 | 0.003 | | |
| | ($\log_n$ [C1 HV3 $\log_{10}$ cfu/ml])$^{2}$ | 0.48 | | | 0.003 | 7.11 | 0.068 |
| *Linear* | A1 HV4 $\log_{10}$ cfu/ml | 0.23 | 6.17 | 1.8 | 0.021 | | |
| *Polynomial* | [A1 HV4 $\log_{10}$ cfu/ml]$^{-2}$ | 0.0016 | 3.02 | 3.7 | 0.003 | | |
| | $\log_n$ [A1 HV4 $\log_{10}$ cfu/ml]$^{1}$ | 2.80 | | | 0.002 | 5.68 | 0.128 |

**Figure A3.1: $Log_{10}$ cfu/ml vs clinical score for the 5 most prevalent *S. zooepidemicus* types including plotted regression line from the best-fitting, 2-power polynomial model**



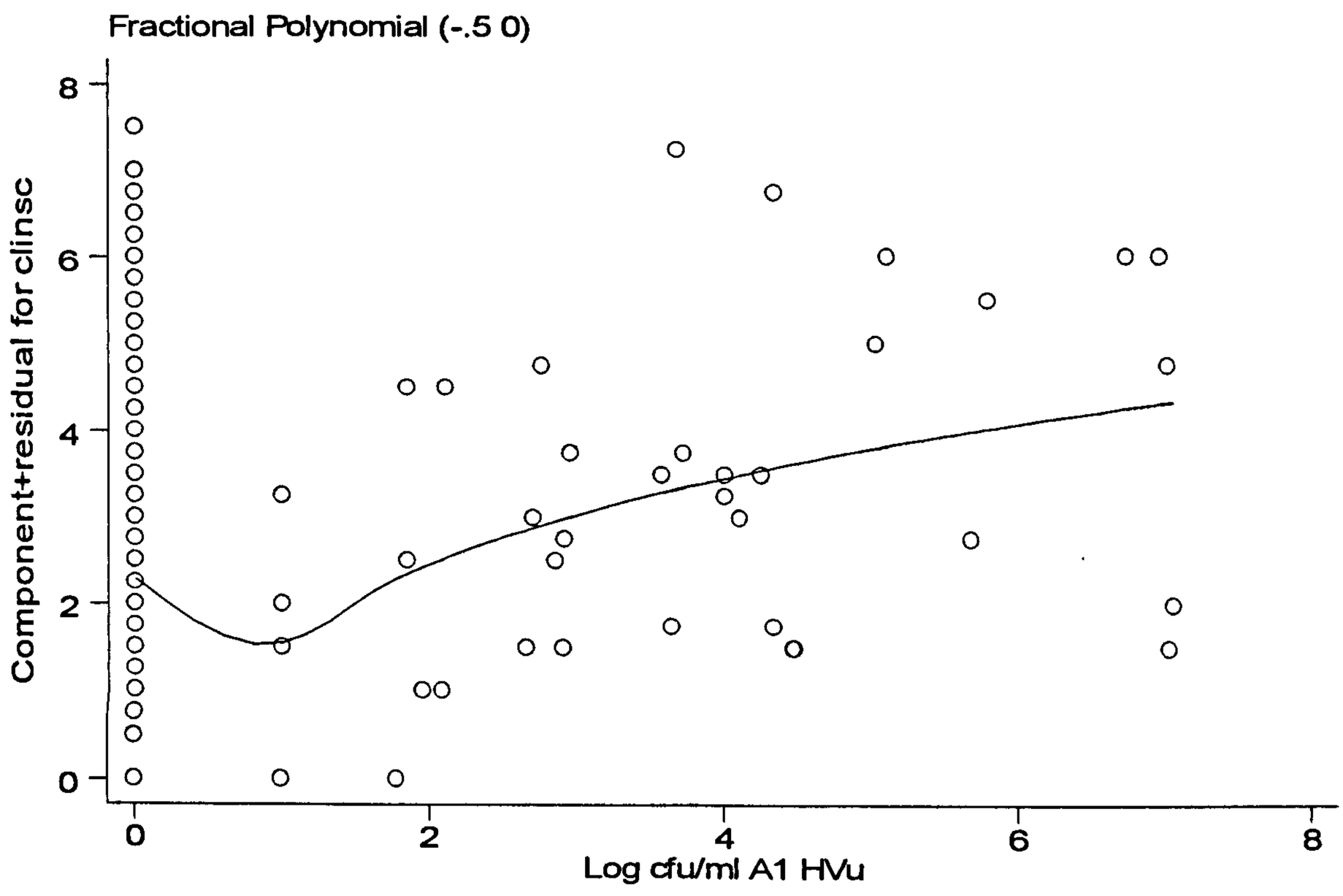
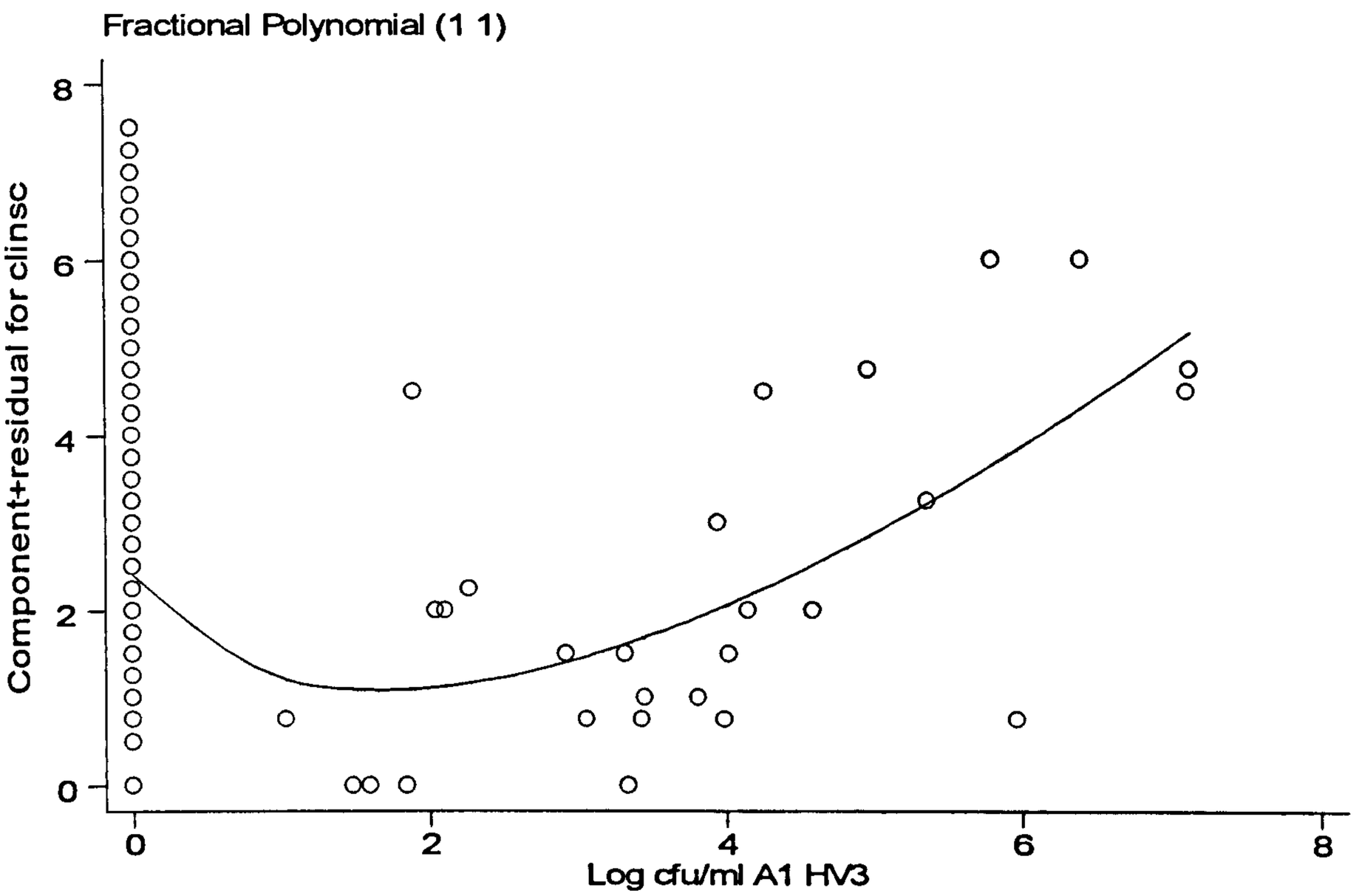



Figure A3.1 continued



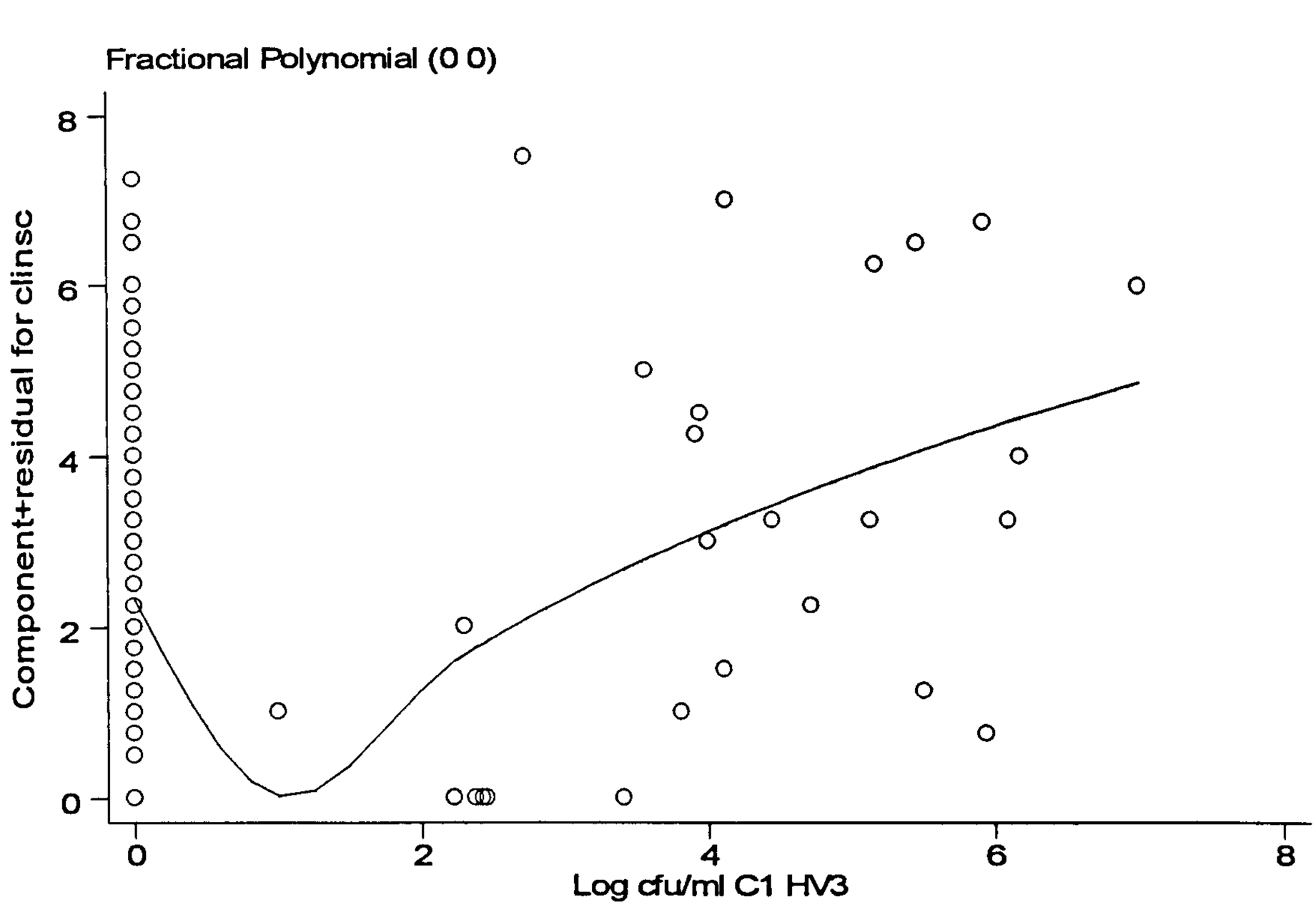



Figure A3.1 continued

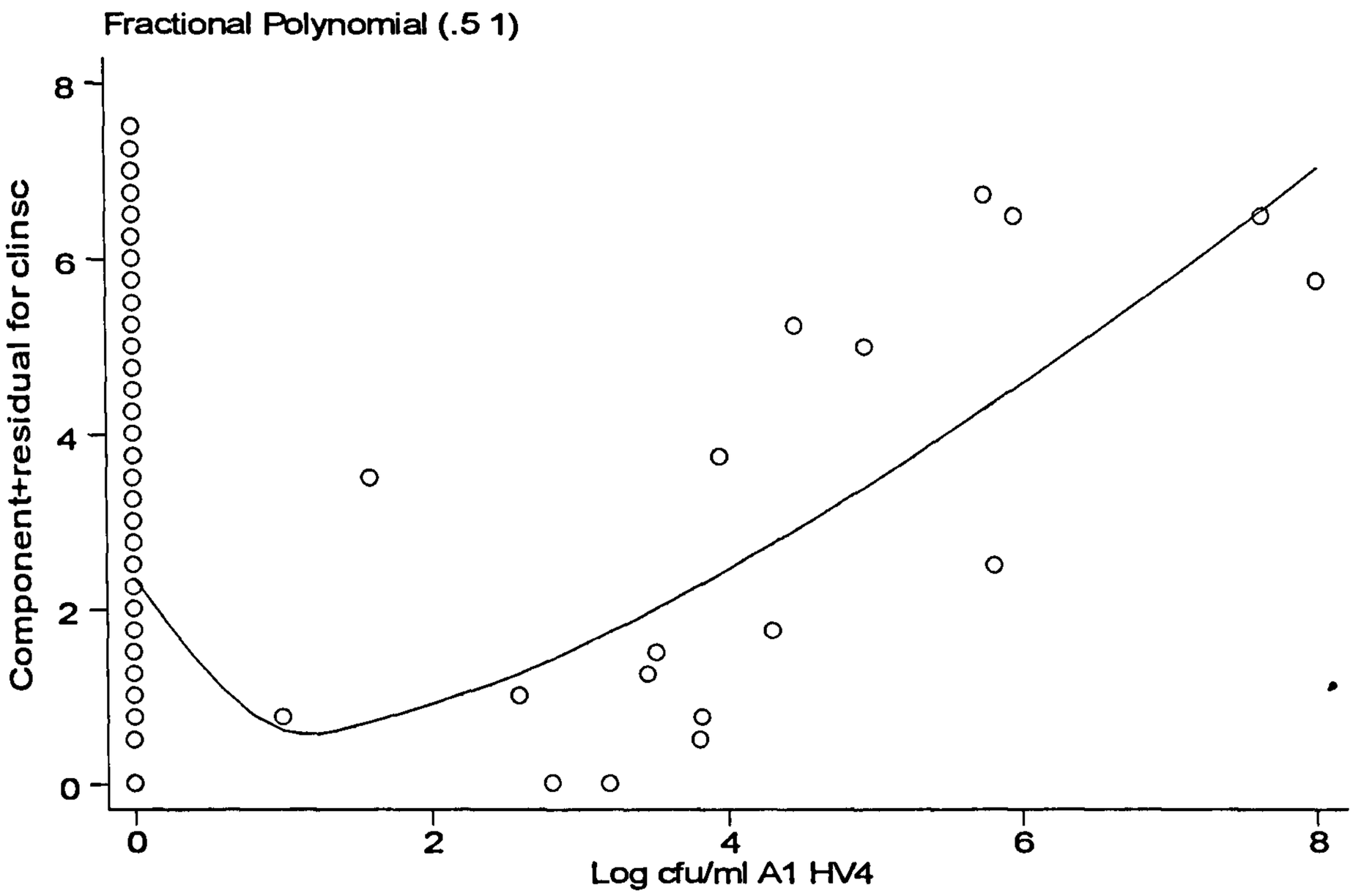
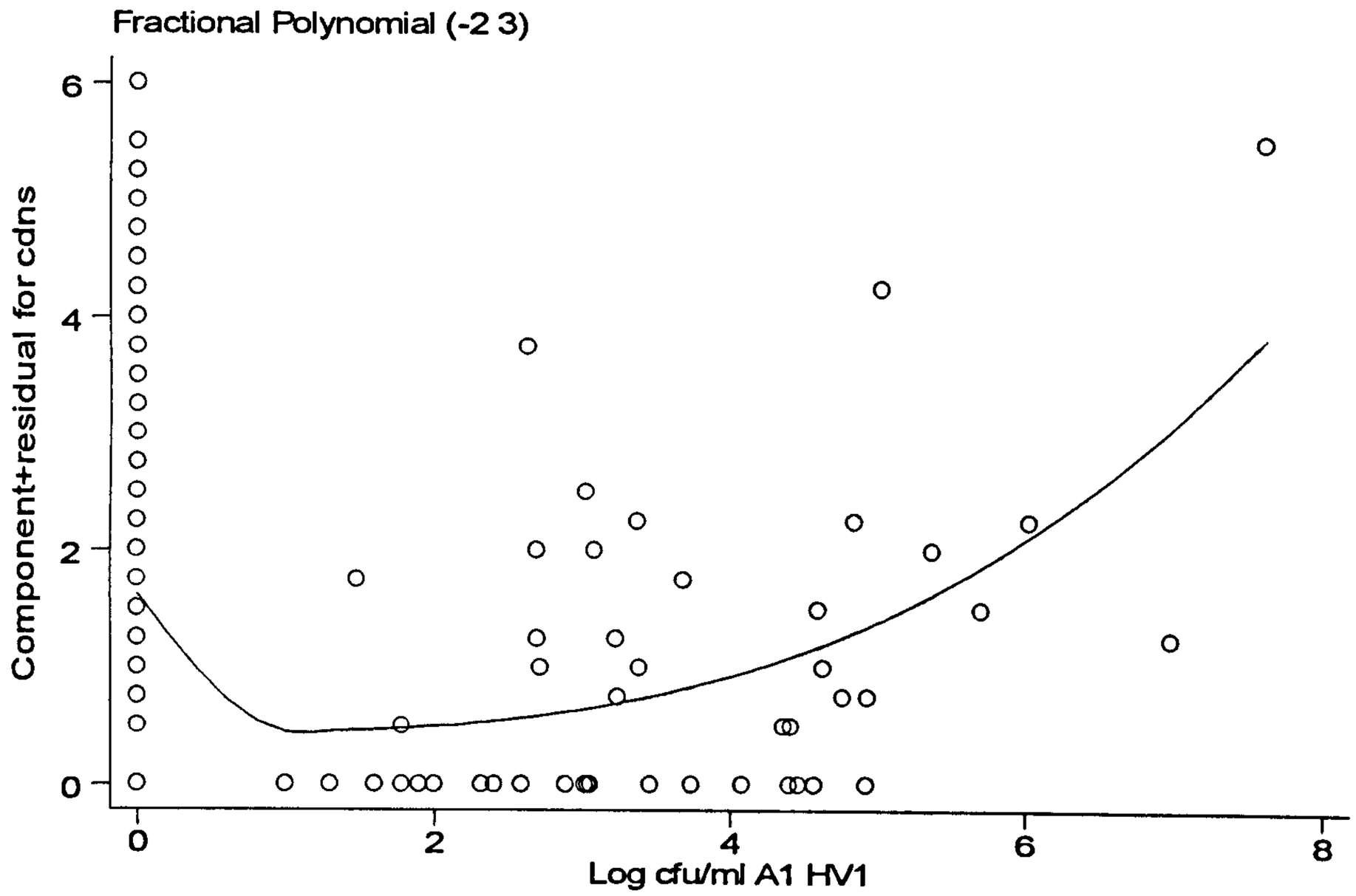


**Figure A3.2: $Log_{10}$ cfu/ml vs CDNS score for the 5 most prevalent *S. zooepidemicus* types including plotted regression line from the best-fitting, 2-power polynomial model**



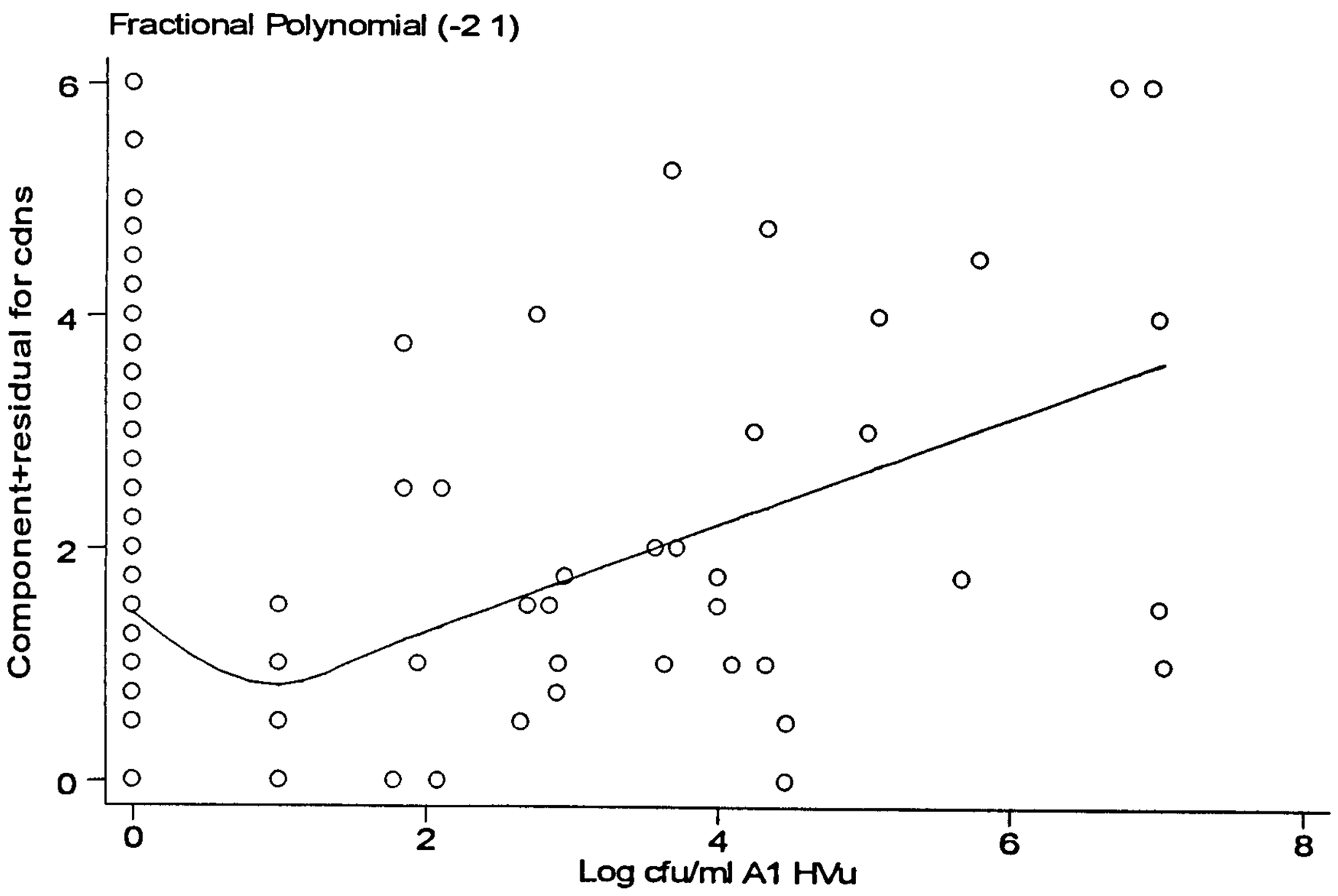
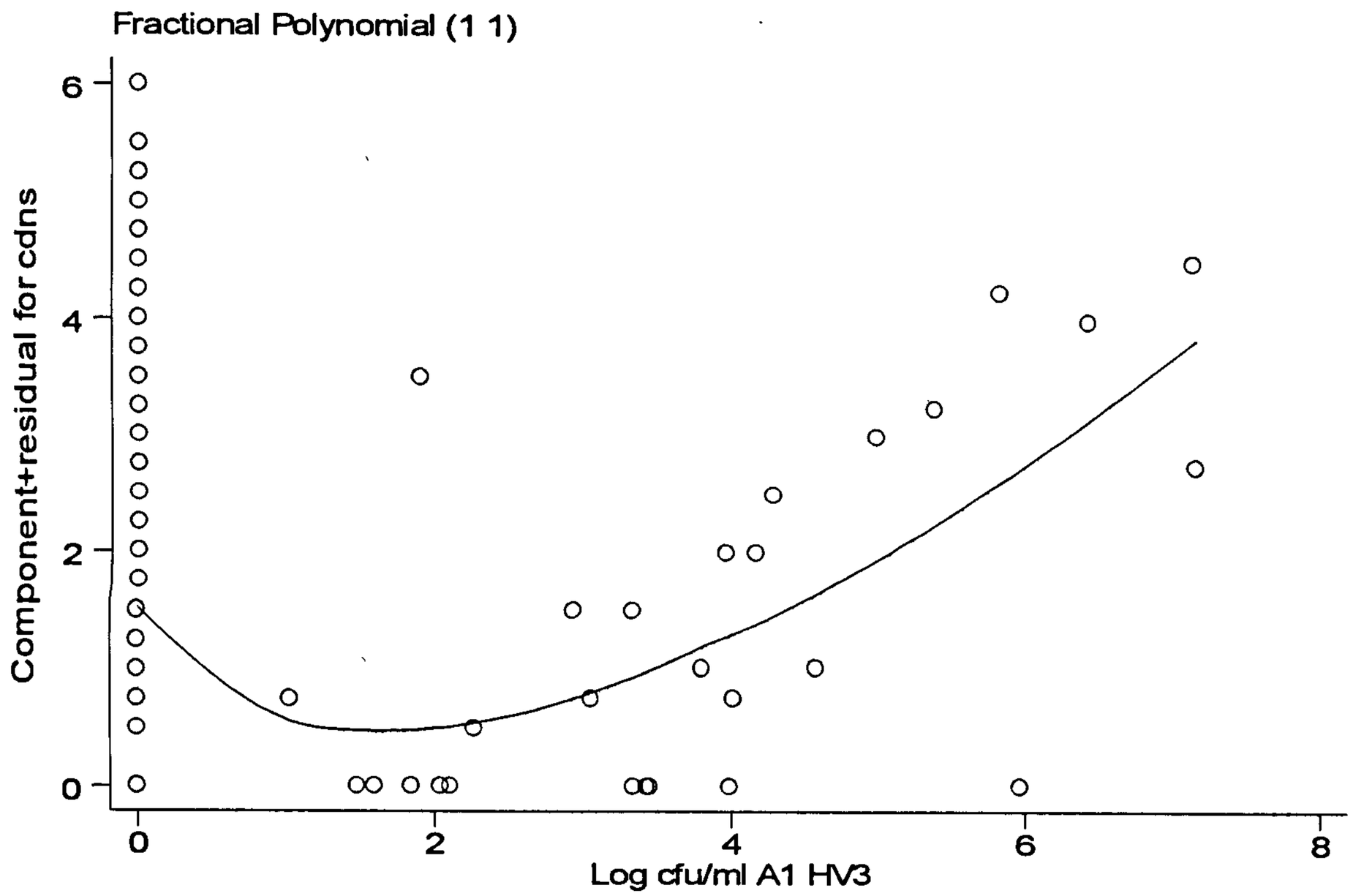



Figure A3.2 continued



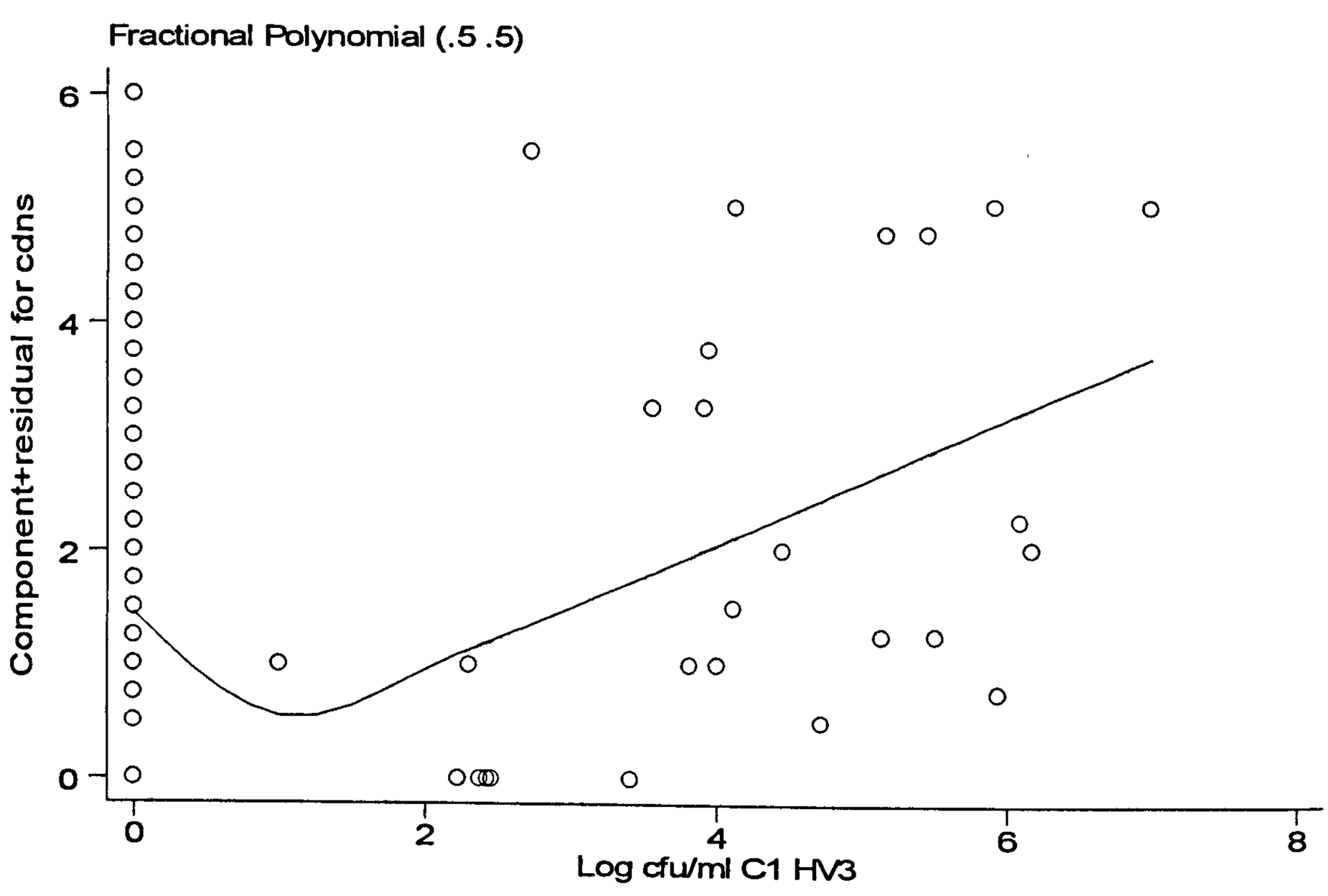



Figure A3.2 continued

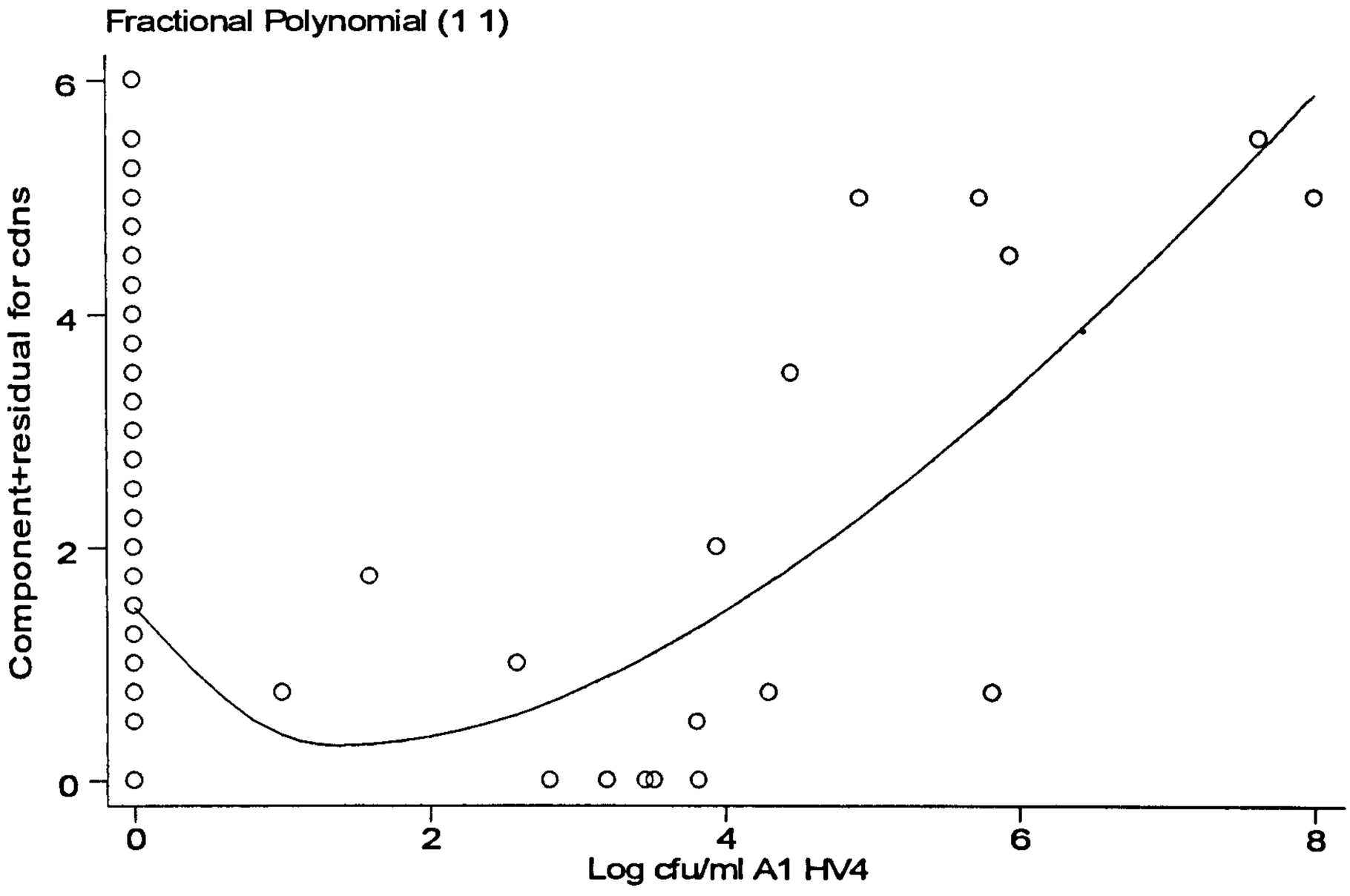


**Figure A3.3: $Log_{10}$ cfu/ml vs airway inflammation score for the 5 most prevalent *S. zooepidemicus* types including plotted regression line from the best-fitting, 2-power polynomial model**

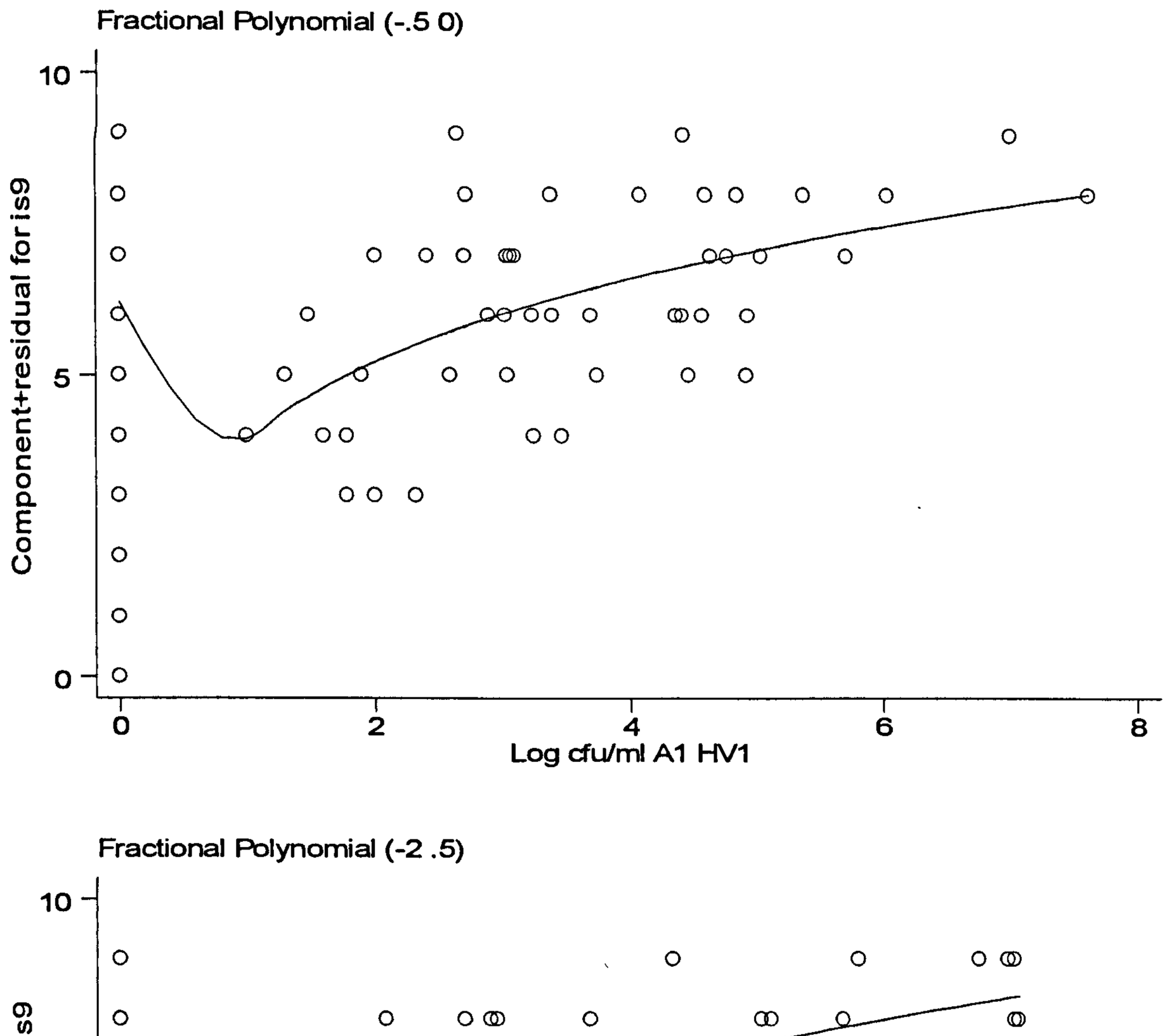
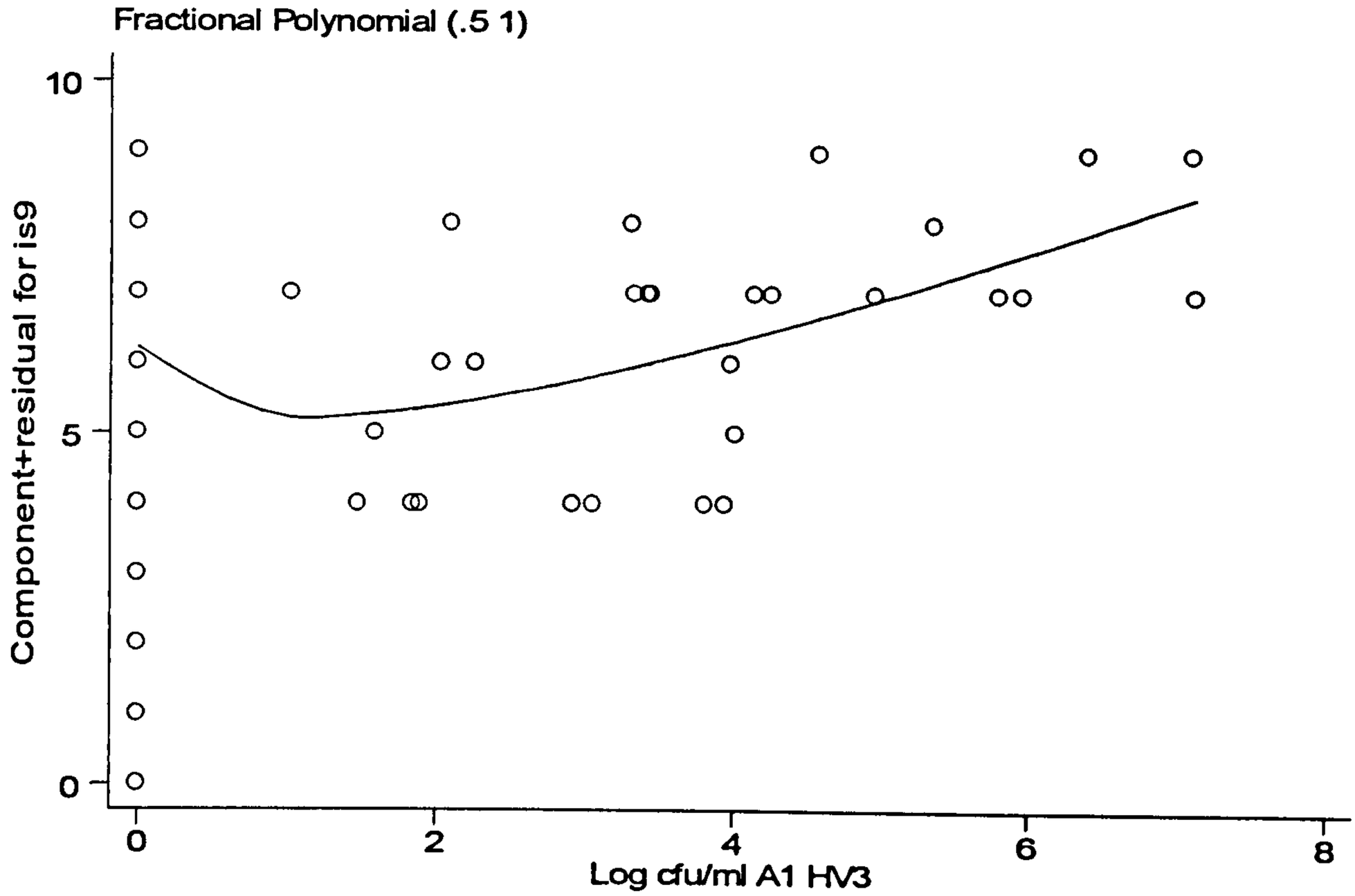


Figure A3.3 continued



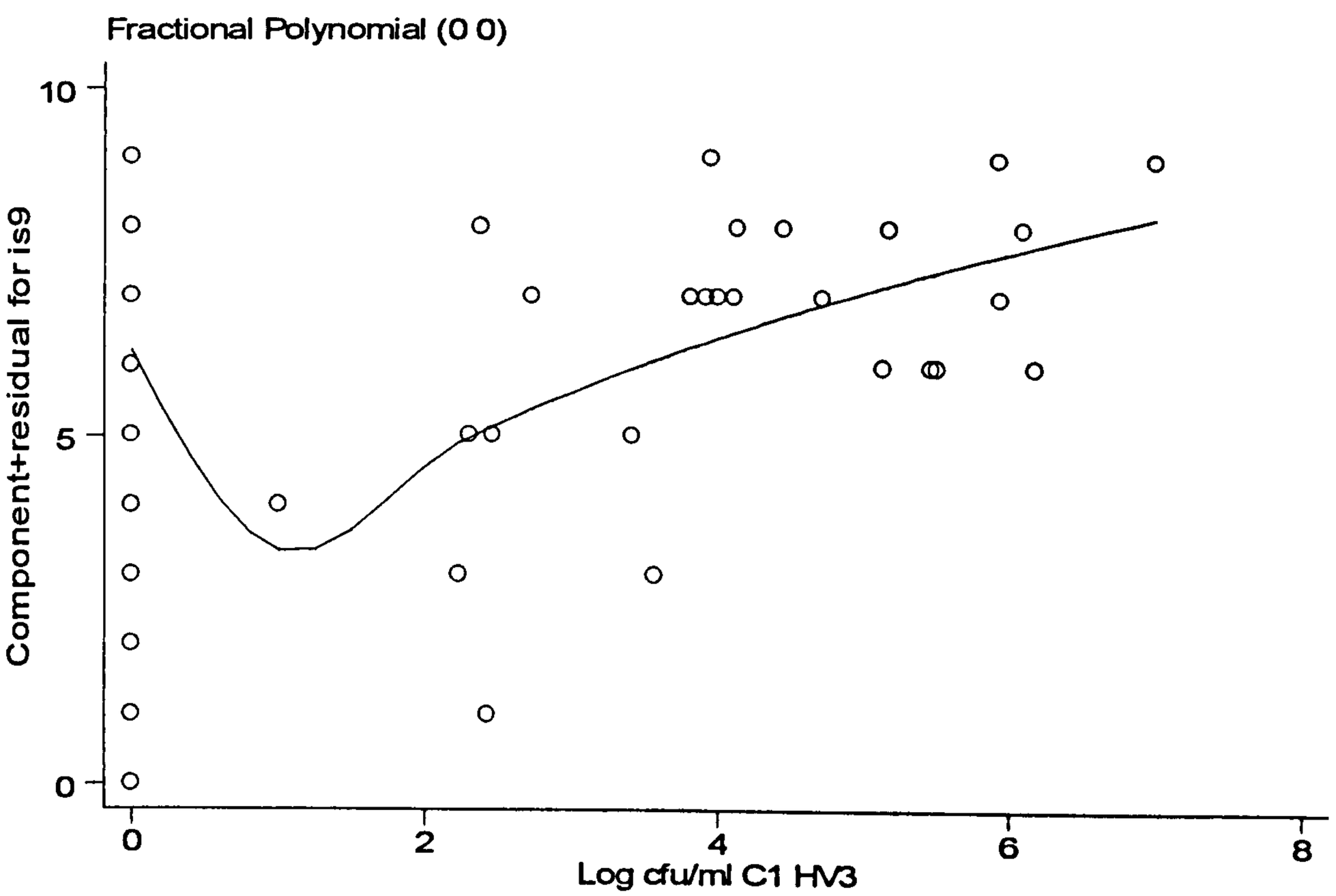
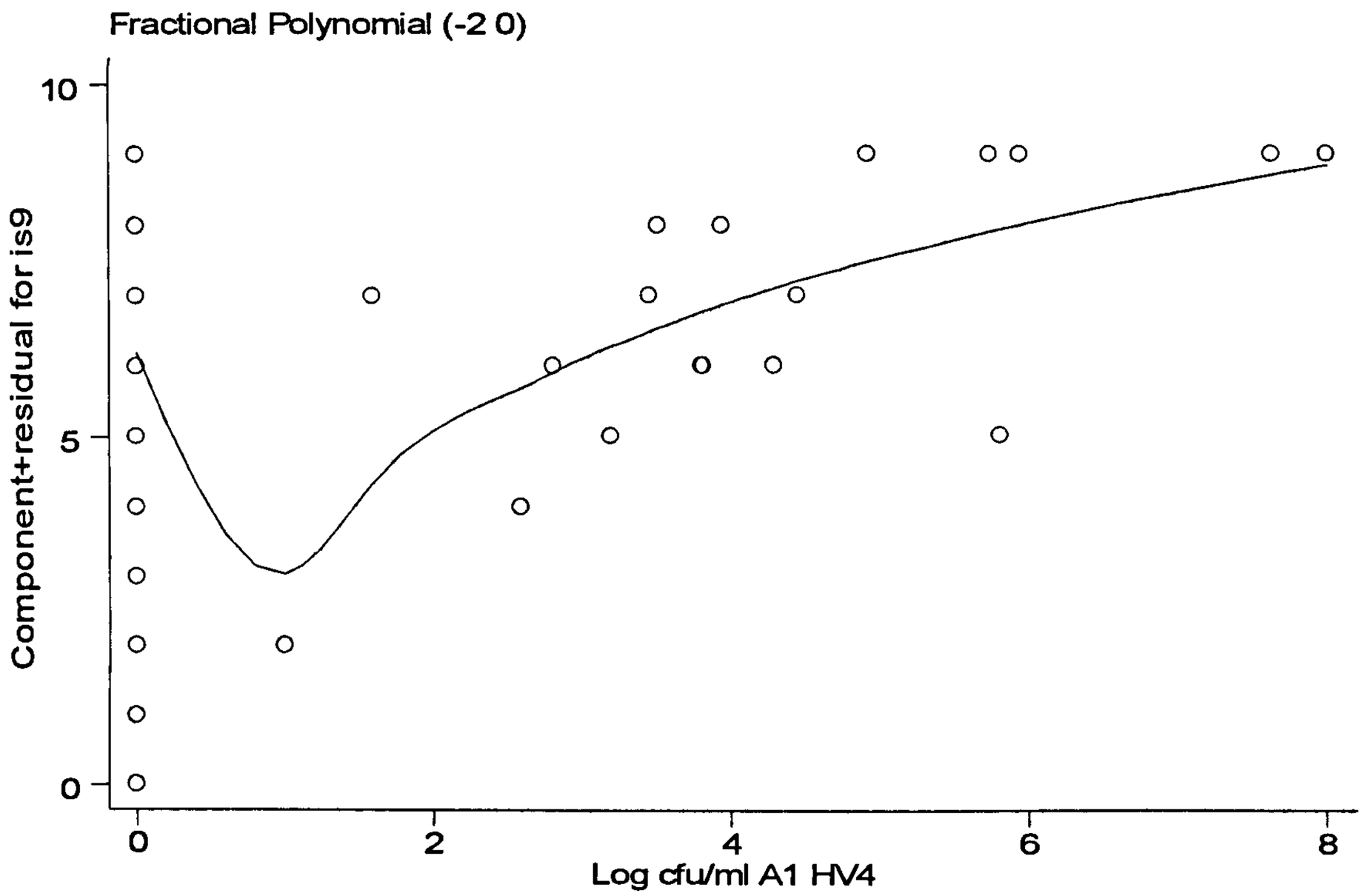



Figure A3.2 continued



# Table A3.2: Summary of multilevel linear regression modelling of clinical and CDNS scores, including individual terms for *S. zooepidemicus* types

| Outcome variable / Effect type / Explanatory variable in model | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) |
|---|---|---|---|---|---|---|
| **Clinical score** | **Model 1 n=218** | **Model 2 n=218** | **Model 3 n=218** | **Model 4 n=218** | **Model 5a n=218** | **Model 5b n=218** |
| Fixed effect | | | | | | |
| Intercept | 2.26 (0.34) | 2.07 (0.36) | 1.76 (0.35) | 6.73 (1.73) | 7.28 (1.73) | 6.26 (1.71) |
| Clinical score 1 week previously | 0.42 (0.07) | 0.41 (0.07) | 0.44 (0.06) | 0.40 (0.06) | 0.38 (0.06) | 0.38 (0.06) |
| Clinical score 2 weeks previously | 0.12 (0.06) | 0.12 (0.06) | 0.14 (0.06) | 0.14 (0.06) | 0.13 (0.06) | 0.12 (0.06) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.68 (0.17) | -0.55 (0.18) | -0.49 (0.17) | -0.47 (0.17) | -0.48 (0.17) | -0.46 (0.17) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.10 (0.03) | 0.08 (0.03) | 0.08 (0.03) | 0.07 (0.03) | 0.07 (0.03) | 0.08 (0.03) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.47 (0.15) | -0.48 (0.14) | -0.46 (0.14) | -0.40 (0.14) | -0.38 90.14) | -0.39 (0.14) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp$]^2$ | 0.08 (0.03) | 0.08 (0.03) | 0.08 (0.02) | 0.07 (0.02) | 0.06 (0.02) | 0.07 (0.02) |
| $Log_{10}$ cfu/ml A1 HVu | -1.10 (0.55) | -1.02 (0.54) | -1.04 (0.53) | -0.97 (0.52) | -0.93 (0.52) | -0.90 (0.52) |
| $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.57 (0.23) | 0.53 (0.23) | 0.55 (0.23) | 0.53 (0.22) | 0.52 (0.22) | 0.50 (0.22) |
| $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.06 (0.02) | -0.06 (0.02) | -0.06 (0.02) | -0.06 (0.02) | -0.06 (0.02) | -0.05 (0.02) |
| $Log_{10}$ cfu/ml A1 HV3 | | -0.37 (0.26) | -0.39 (0.25) | -0.37 (0.25) | -0.35 (0.25) | -0.36 (0.24) |
| $[Log_{10}$ cfu/ml A1 HV3$]^2$ | | 0.09 (0.05) | 0.09 (0.05) | 0.09 (0.04) | 0.09 (0.04) | 0.09 (0.04) |
| $Log_{10}$ cfu/ml C1 HV3 | | | 0.22 (0.07) | 0.22 (0.07) | 0.22 (0.07) | 0.21 (0.07) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-1}$ | | | | 0.96 (0.33) | 0.98 (0.33) | 0.90 (0.33) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | | | | -10.1 (3.47) | -10.3 (3.45) | -9.48 (3.41) |
| Transferrin D phenotype | | | | | -0.53 (0.23) | |
| Transferrin F2 phenotype | | | | | | 0.52 (0.25) |
| Random effect | | | | | | |
| Level 2 variance: Pony | 0.12 (0.09) | 0.17 (0.11) | 0.07 (0.08) | 0.14 (0.09) | 0.09 (0.08) | 0.14 (0.09) |
| Level 1 variance: Observation | 1.75 (0.18) | 1.66 (0.17) | 1.65 (0.17) | 1.55 (0.16) | 1.54 (0.16) | 1.52 (0.16) |
| *-2*loglikelihood* | *751.84* | *745.74* | *736.56* | *728.52* | *723.16* | *724.28* |
| *LRS $\chi^2$ (d.f.)* | | *6.10 (2)* | *9.18 (1)* | *8.04 (2)* | *5.36 (1)* | *4.24 (1)* |
| *P-value* | | *0.048* | *0.002* | *0.018* | *0.021* | *0.040* |
| *Intra-pony correlation (%)* | *6.3* | *9.4* | *4.2* | *8.2* | *5.8* | *8.2* |
| **CDNS score** | **Model 1 n=244** | **Model 2 n=244** | **Model 3 n=244** | **Model 4 n=244** | **Model 5a n=244** | **Model 5b n=244** |
| Fixed effect | | | | | | |
| Intercept | 1.80 (0.25) | 1.67 (0.26) | 1.54 (0.26) | 4.84 (1.34) | 5.13 (1.33) | 4.42 (1.34) |
| CDNS score 1 week previously | 0.45 (0.05) | 0.45 (0.05) | 0.45 (0.05) | 0.43 (0.05) | 0.42 (0.05) | 0.42 (0.05) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.51 (0.13) | -0.42 (0.14) | -0.38 (0.14) | -0.40 (0.14) | -0.40 (0.14) | -0.38 (0.14) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.07 (0.02) | 0.05 (0.02) | 0.05 (0.02) | 0.05 (0.02) | 0.05 (0.02) | 0.05 (0.02) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.39 (0.11) | -0.39 (0.11) | -0.38 (0.11) | -0.34 (0.11) | -0.33 (0.11) | -0.34 (0.11) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp$]^2$ | 0.07 (0.02) | 0.07 (0.02) | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) |
| $Log_{10}$ cfu/ml A1 Hvu | -0.64 (0.44) | -0.61 (0.44) | -0.57 (0.43) | -0.60 (0.43) | -0.59 (0.43) | -0.58 (0.42) |
| $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.36 (0.19) | 0.35 (0.19) | 0.34 (0.19) | 0.34 (0.18) | 0.34 (0.18) | 0.34 (0.18) |
| $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.04 (0.02) | -0.04 (0.02) | 0.04 (0.02) | -0.04 (0.02) | -0.04 (0.02) | -0.04 (0.02) |
| $Log_{10}$ cfu/ml A1 HV3 | | -0.30 (0.20) | -0.30 (0.20) | -0.30 (0.20) | -0.30 (0.19) | -0.31 (0.19) |
| $[Log_{10}$ cfu/ml A1 HV3$]^2$ | | 0.07 (0.04) | 0.07 (0.04) | 0.07 (0.04) | 0.07 (0.04) | 0.07 (0.04) |
| $Log_{10}$ cfu/ml C1 HV3 | | | 0.12 (0.06) | 0.12 (0.06) | 0.12 (0.06) | 0.11 (0.06) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-2}$ | | | | 0.01 (0.002) | 0.01 (0.002) | 0.01 (0.002) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | | | | -6.90 (2.54) | -6.86 (2.51) | -6.56 (2.52) |
| Transferrin D phenotype | | | | | -0.45 (0.21) | |
| Transferrin F2 phenotype | | | | | | 0.48 (0.21) |
| Random effect | | | | | | |
| Level 2 variance: Pony | 0.16 (0.08) | 0.19 (0.08) | 0.17 (0.08) | 0.20 (0.09) | 0.15 (0.07) | 0.15 (0.07) |
| Level 1 variance: Observation | 1.14 (0.11) | 1.10 (0.11) | 1.08 (0.10) | 1.04 (0.10) | 1.04 (0.10) | 1.03 (0.10) |
| *-2*loglikelihood* | *746.33* | *740.85* | *736.31* | *728.52* | *723.96* | *723.47* |
| *LRS $\chi^2$ (d.f.)* | | *5.48 (2)* | *4.54 (1)* | *7.79 (2)* | *4.56 (1)* | *5.05 (1)* |
| *P-value* | | *0.065* | *0.033* | *0.020* | *0.033* | *0.025* |
| *Intra-pony correlation (%)* | *12.3* | *14.4* | *13.9* | *16.0* | *12.4* | *12.8* |

# Table A3.3: Summary of multilevel linear regression modelling of airway inflammation (AI) score, including individual terms for *S. zooepidemicus* types

| Outcome variable / Effect type / Explanatory variable in model | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) |
|---|---|---|---|---|---|
| **Airway inflammation score** | **Model 1** n=225 | **Model 2** n=225 | **Model 3** n=214 | **Model 4** n=214 | **Model 5** n=214 |
| Fixed effect | | | | | |
| Intercept | 2.81 (0.42) | 3.78 (0.51) | 6.36 (1.03) | 3.65 (1.93) | 3.63 (1.91) |
| AI 1 week previously | 0.34 (0.06) | 0.32 (0.06) | 0.30 (0.06) | 0.31 (0.06) | 0.30 (0.06) |
| AI 2 weeks previously | 0.25 (0.06) | 0.22 (0.05) | 0.20 (0.05) | 0.20 (0.05) | 0.21 (0.05) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | | -0.74 (0.16) | -0.73 (0.16) | -0.67 (0.17) | -0.59 (0.17) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | | 0.12 (0.03) | 0.11 (0.03) | 0.10 (0.03) | 0.09 (0.03) |
| $[Log_{10}$ cfu/ml A1 HVu$]^{-1}$ | | | 0.39 (0.15) | 0.39 (0.14) | 0.40 (0.14) |
| $[Log_{10}$ cfu/ml A1 HVu$]^{-0.5}$ | | | -4.09 (1.54) | -4.17 (1.52) | -4.26 (1.51) |
| $[Log_{10}$ cfu/ml C1 HV3$]^{-0.5}$ | | | | 1.08 (0.59) | 1.11 (0.59) |
| $Log_n$ [$Log_{10}$ cfu/ml C1 HV3] | | | | 1.77 (0.93) | 1.83 (0.92) |
| $Log_{10}$ cfu/ml A1 HV4 | | | | | 0.16 (0.08) |
| Random effect | | | | | |
| Level 2 variance: Pony | 0.03 (0.09) | 0.004 (0.08) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| Level 1 variance: Observation | 2.36 (0.24) | 2.19 (0.22) | 2.00 (0.19) | 1.96 (0.19) | 1.92 (0.19) |
| *-2*loglikelihood* | *834.63* | *815.22* | *756.05* | *751.08* | *747.14* |
| *LRS $\chi^2$ (d.f.)* | | *19.41 (2)* | | *4.97 (2)* | *3.94 (1)* |
| *P-value* | | *<0.0001* | | *0.083* | *0.047* |
| *Intra-pony correlation (%)* | *1.3* | *0.2* | *0.0* | *0.0* | *0.0* |

**Figure A3.4: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for clinical score, including transferrin D haplotype (Model 5a)**

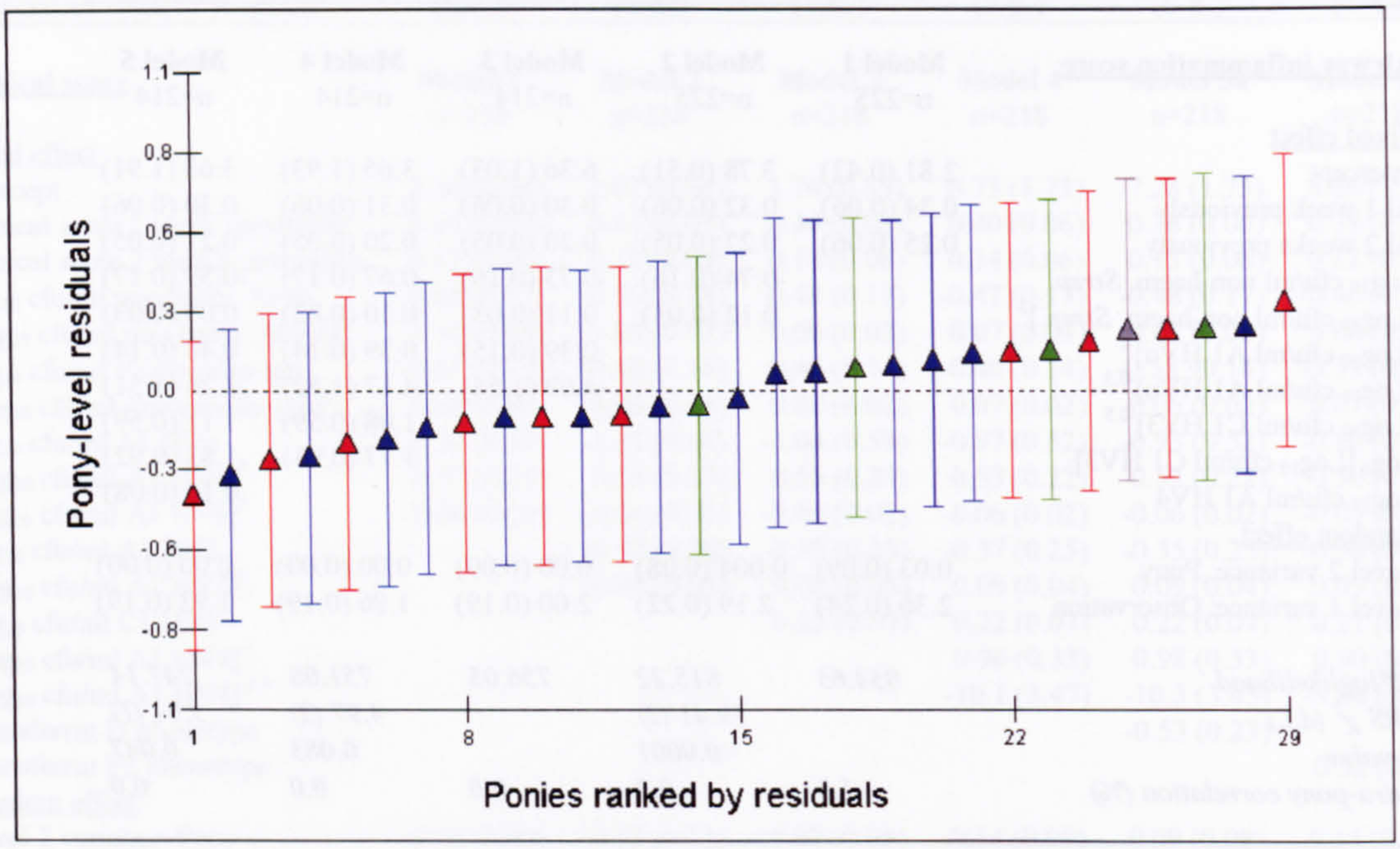
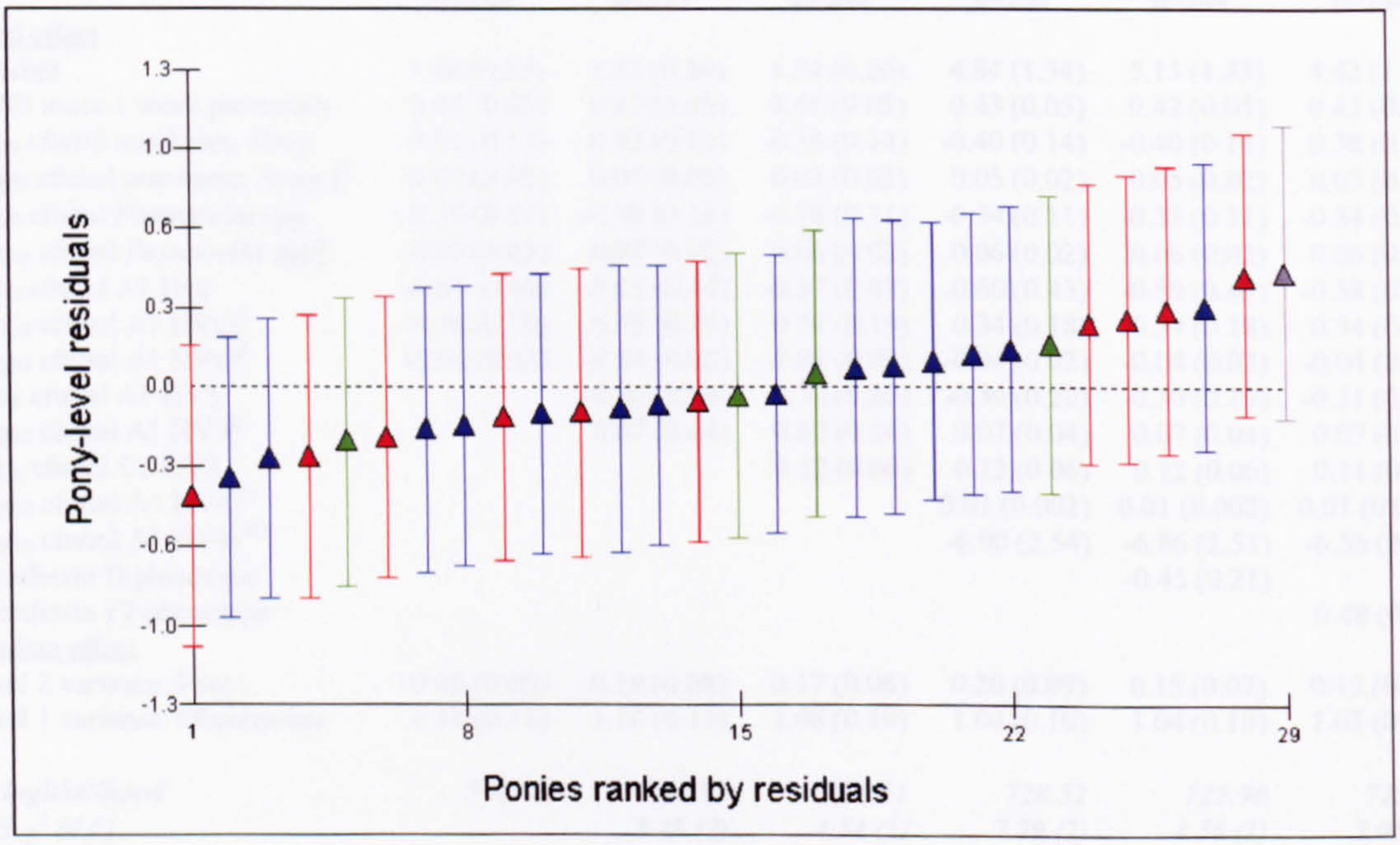



**Figure A3.5: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for clinical score, including transferrin F2 haplotype (Model 5b)**




Note: Colour scheme represents the transferrin D/F2 phenotype status of each pony light blue: transferrin D phenotype (n=14), red: transferrin F2 phenotype (n=10), light green: transferrin DF2 phenotype (n=4) & grey: other transferrin phenotype (n=1)

**Figure A3.6: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for CDNS score, including transferrin D haplotype (Model 5a)**

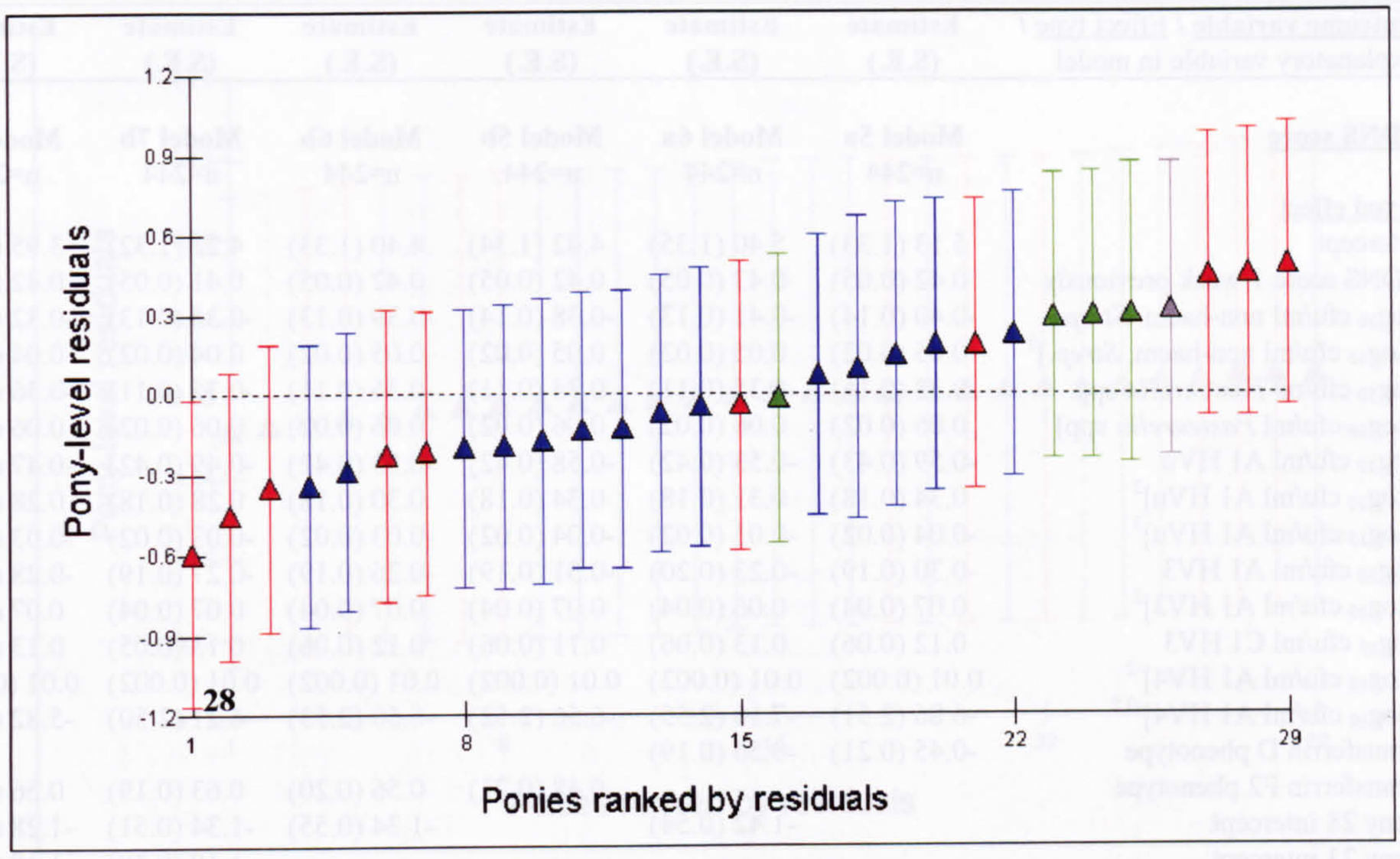
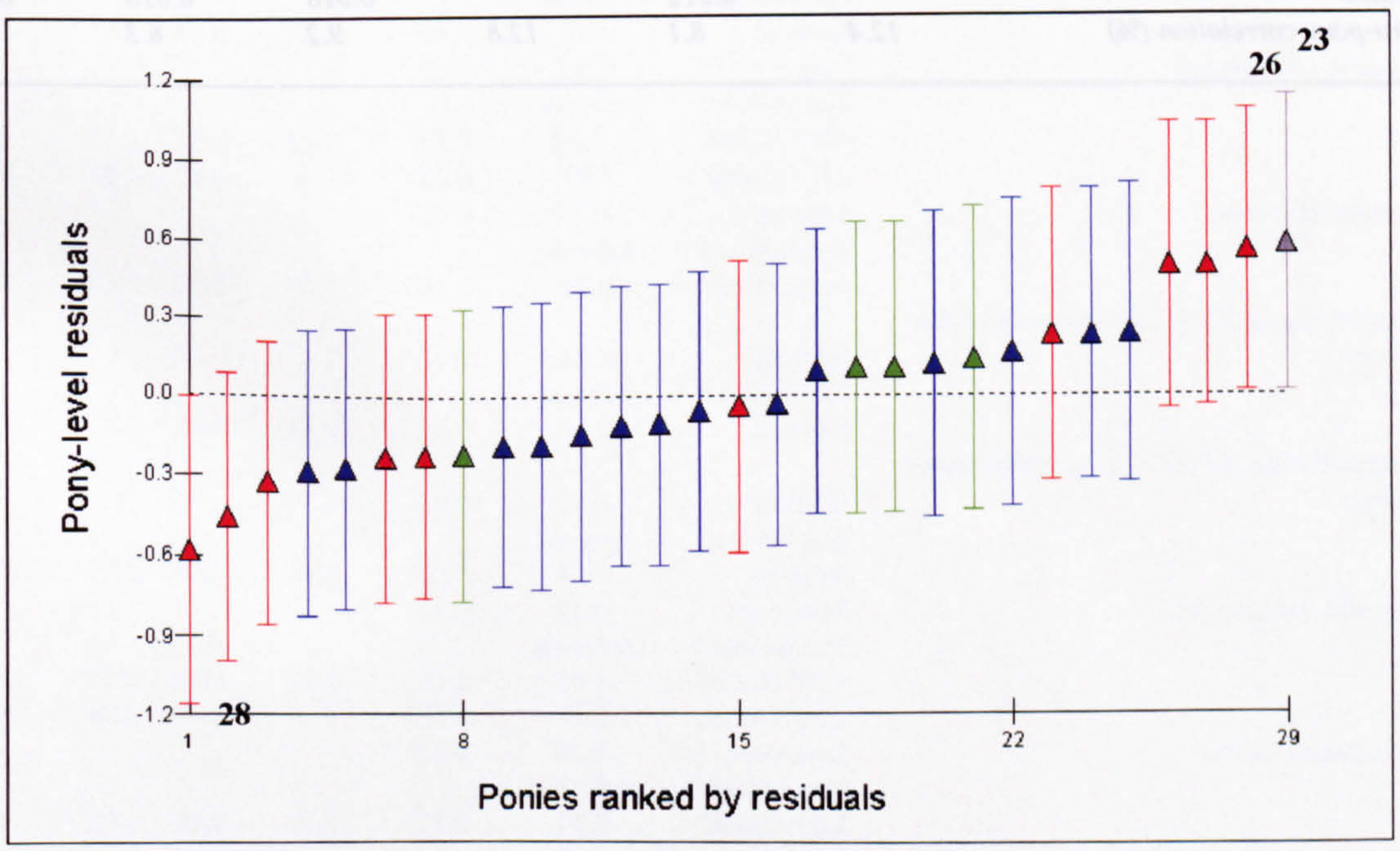



**Figure A3.7: Caterpillar plot of ranked pony level residuals (± 95% CI) for final multilevel linear regression model for CDNS score, including transferrin F2 haplotype (Model 5b)**

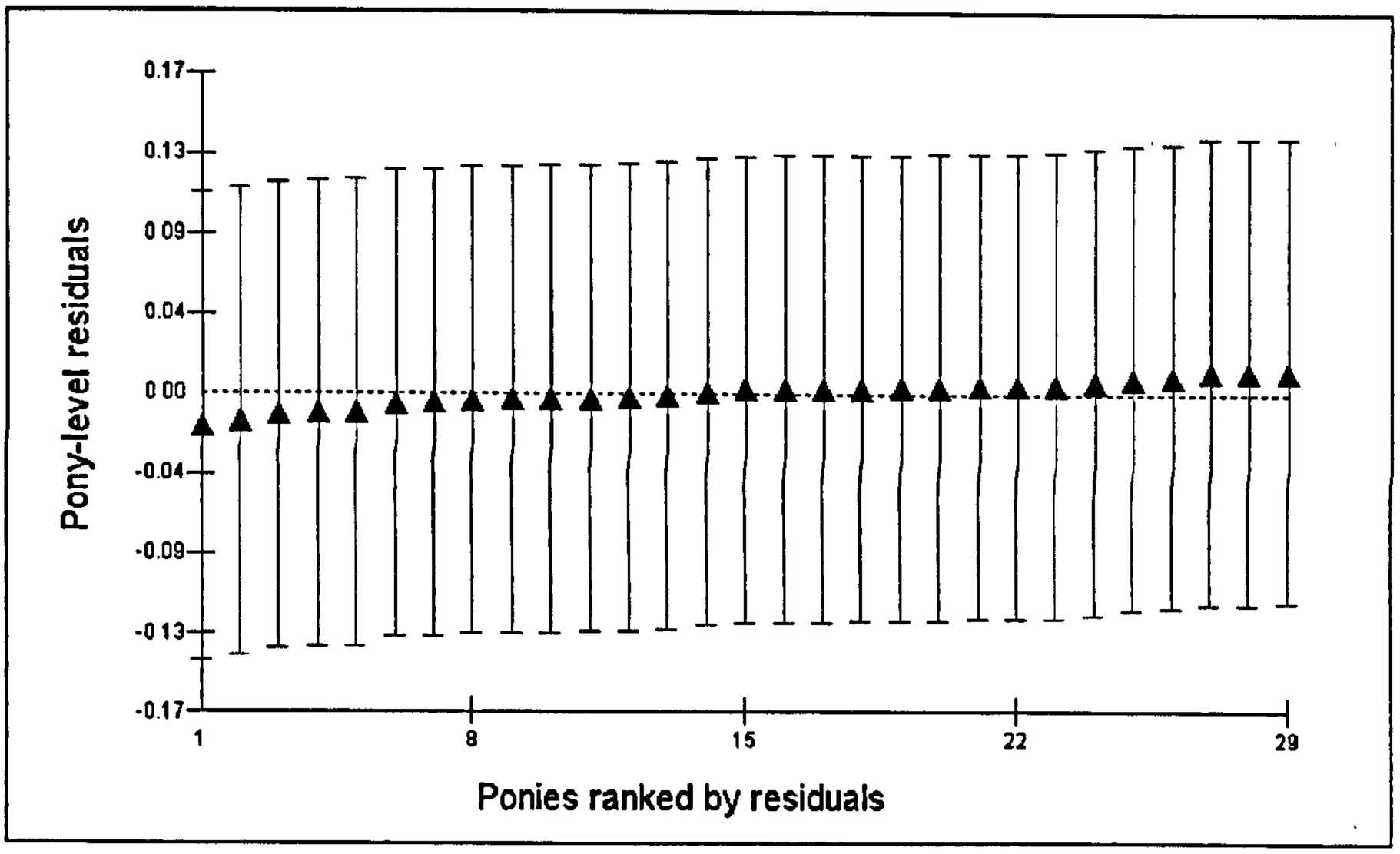



Note: Colour scheme represents the transferrin D/F2 phenotype status of each pony light blue: transferrin D phenotype (n=14), red: transferrin F2 phenotype (n=10), light green: transferrin DF2 phenotype (n=4) & grey: other transferrin phenotype (n=1)

# Table A3.4: Summary of multilevel linear regression modelling of CDNS scores, including individual terms for *S. zooepidemicus* types and with sequential exclusion from random effects components of ponies with largest value residuals

| Outcome variable / Effect type / Explanatory variable in model | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) | Estimate (S.E.) |
|---|---|---|---|---|---|---|
| **CDNS score** | **Model 5a n=244** | **Model 6a n=244** | **Model 5b n=244** | **Model 6b n=244** | **Model 7b n=244** | **Model 8b n=244** |
| Fixed effect | | | | | | |
| Intercept | 5.13 (1.33) | 5.40 (1.35) | 4.42 (1.34) | 4.40 (1.33) | 4.22 (1.32) | 3.95 (1.30) |
| CDNS score 1 week previously | 0.42 (0.05) | 0.43 (0.05) | 0.42 (0.05) | 0.42 (0.05) | 0.41 (0.05) | 0.42 (0.05) |
| $Log_{10}$ cfu/ml non-haem. *Strep.* | -0.40 (0.14) | -0.41 (0.13) | -0.38 (0.14) | -0.39 (0.13) | -0.35 (0.13) | -0.32 (0.13) |
| $[Log_{10}$ cfu/ml non-haem. *Strep.*$]^2$ | 0.05 (0.02) | 0.05 (0.02) | 0.05 (0.02) | 0.05 (0.02) | 0.04 (0.02) | 0.04 (0.02) |
| $Log_{10}$ cfu/ml *Pasteurella* spp. | -0.33 (0.11) | -0.35 (0.11) | -0.34 (0.11) | -0.36 (0.11) | -0.35 (0.11) | -0.36 (0.10) |
| $[Log_{10}$ cfu/ml *Pasteurella* spp$]^2$ | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) | 0.06 (0.02) |
| $Log_{10}$ cfu/ml A1 HVu | -0.59 (0.43) | -0.53 (0.42) | -0.58 (0.42) | -0.53 (0.42) | -0.49 (0.42) | -0.47 (0.42) |
| $[Log_{10}$ cfu/ml A1 HVu$]^2$ | 0.34 (0.18) | 0.31 (0.18) | 0.34 (0.18) | 0.30 (0.18) | 0.28 (0.18) | 0.28 (0.18) |
| $[Log_{10}$ cfu/ml A1 HVu$]^3$ | -0.04 (0.02) | -0.03 (0.02) | -0.04 (0.02) | -0.03 (0.02) | -0.03 (0.02) | -0.03 (0.02) |
| $Log_{10}$ cfu/ml A1 HV3 | -0.30 (0.19) | -0.23 (0.20) | -0.31 (0.19) | -0.26 (0.19) | -0.27 (0.19) | -0.28 (0.19) |
| $[Log_{10}$ cfu/ml A1 HV3$]^2$ | 0.07 (0.04) | 0.06 (0.04) | 0.07 (0.04) | 0.07 (0.04) | 0.07 (0.04) | 0.07 (0.04) |
| $Log_{10}$ cfu/ml C1 HV3 | 0.12 (0.06) | 0.13 (0.06) | 0.11 (0.06) | 0.12 (0.06) | 0.13 (0.05) | 0.13 (0.05) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-2}$ | 0.01 (0.002) | 0.01 (0.002) | 0.01 (0.002) | 0.01 (0.002) | 0.01 (0.002) | 0.01 (0.002) |
| $[Log_{10}$ cfu/ml A1 HV4$]^{-0.5}$ | -6.86 (2.51) | -7.10 (2.55) | -6.56 (2.52) | -6.50 (2.53) | -6.21 (2.50) | -5.82 (2.47) |
| Transferrin D phenotype | -0.45 (0.21) | -0.56 (0.19) | | | | |
| Transferrin F2 phenotype | | | 0.48 (0.21) | 0.56 (0.20) | 0.63 (0.19) | 0.56 (0.17) |
| Pony 28 intercept | | -1.42 (0.54) | | -1.34 (0.55) | -1.34 (0.51) | -1.28 (0.48) |
| Pony 23 intercept | | | | | 1.19 (0.49) | 1.18 (0.45) |
| Pony 26 intercept | | | | | | 0.97 (0.41) |
| Random effect | | | | | | |
| Level 2 variance: Pony | 0.15 (0.07) | 0.09 (0.06) | 0.15 (0.07) | 0.11 (0.06) | 0.07 (0.05) | 0.04 (0.04) |
| Level 1 variance: Observation | 1.04 (0.10) | 1.04 (0.10) | 1.03 (0.10) | 1.03 (0.10) | 1.03 (0.10) | 1.03 (0.10) |
| *-2*loglikelihood* | *723.96* | *717.66* | *723.47* | *717.87* | *712.24* | *707.18* |
| *LRS $\chi^2$ (d.f.)* | | *6.30 (1)* | | *5.60 (1)* | *5.63 (1)* | *5.06 (1)* |
| *P-value* | | *0.012* | | *0.018* | *0.018* | *0.024* |
| *Intra-pony correlation (%)* | *12.4* | *8.1* | *12.8* | *9.2* | *6.3* | *3.3* |

**Figure A3.8: Caterpillar plot of ranked pony level residuals (± 95% CI) for multilevel linear regression model for airway inflammation score including 2 autoregressive variables and a quadratic term for non-haemolytic *Streptococcus* spp. (Model 2)**




Note: Colour scheme represents the transferrin D/F2 phenotype status of each pony light blue: transferrin D phenotype (n=14), red: transferrin F2 phenotype (n=10), light green: transferrin DF2 phenotype (n=4) & grey: other transferrin phenotype (n=1)

# Table A3.5: Results of univariable ordinary logistic regression (OLR) analyses of the risk of nasal discharge with the 5 most prevalent *S. zooepidemicus* types

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| Tracheal wash A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.07 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.32 | 0.32 | 0.73 | 0.39 – 1.37 | 0.326 |
| **Ordered categorical** | Intercept | -0.06 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -1.11 | 0.59 | 0.33 | 0.10 – 1.03 | 0.057 |
| | $\geq 10^3$ cfu/ml | 0.06 | 0.39 | 1.07 | 0.50 – 2.28 | 0.867 |
| **Continuous linear** | Intercept | -0.13 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.02 | 0.08 | 1.02 | 0.87 – 1.20 | 0.780 |
| Nasopharyngeal A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.06 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.24 | 0.32 | 0.79 | 0.42 – 1.48 | 0.456 |
| Tracheal wash A1 HVu *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.14 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.14 | 0.34 | 1.15 | 0.59 – 2.23 | 0.685 |
| **Ordered categorical** | Intercept | -0.13 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -0.57 | 0.52 | 0.57 | 0.21 – 1.56 | 0.270 |
| | $\geq 10^3$ cfu/ml | 0.61 | 0.47 | 1.84 | 0.74 – 4.59 | 0.191 |
| **Continuous linear** | Intercept | -0.16 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.10 | 0.09 | 1.11 | 0.94 – 1.31 | 0.229 |
| Nasopharyngeal A1 HVu *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.11 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.06 | 0.34 | 1.06 | 0.55 – 2.04 | 0.866 |
| Tracheal wash A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.12 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.03 | 0.40 | 0.97 | 0.45 – 2.12 | 0.948 |
| **Ordered categorical** | Intercept | -0.11 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -1.14 | 0.81 | 0.32 | 0.07 – 1.57 | 0.160 |
| | $\geq 10^3$ cfu/ml | 0.43 | 0.48 | 1.54 | 0.60 – 3.95 | 0.370 |
| **Continuous linear** | Intercept | -0.14 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.08 | 0.10 | 1.09 | 0.90 – 1.31 | 0.396 |
| Nasopharyngeal A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.10 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.10 | 0.46 | 1.11 | 0.45 – 2.75 | 0.821 |
| **Tracheal wash C1 HV3 *S. zooepidemicus*** | | | | | | |
| **Binary** | Intercept | -0.17 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.64 | 0.42 | 1.90 | 0.83 – 4.35 | 0.126 |
| **Ordered categorical** | **Intercept** | **-0.17** | **0.12** | | | |
| | **Not isolated** | **referent** | | | | |
| | **$<10^3$ cfu/ml** | **-1.62** | **1.09** | **0.20** | **0.02 – 1.67** | **0.136** |
| | **$\geq 10^3$ cfu/ml** | **1.49** | **0.58** | **4.45** | **1.44 – 13.8** | **0.010** |
| **Continuous linear** | **Intercept** | **-0.20** | **0.12** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Log cfu/ml$^{-1}$** | **0.26** | **0.11** | **1.30** | **1.05 – 1.60** | **0.015** |
| Nasopharyngeal C1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.16 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.62 | 0.39 | 1.86 | 0.87 – 3.97 | 0.110 |

Table A3.5 continued

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| Tracheal wash A1 HV4 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.13 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.13 | 0.49 | 1.14 | 0.44 – 2.94 | 0.794 |
| **Ordered categorical** | Intercept | -0.12 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | 0.12 | 1.01 | 1.13 | 0.16 – 8.14 | 0.903 |
| | $\geq 10^3$ cfu/ml | 0.12 | 0.55 | 1.13 | 0.39 – 3.31 | 0.823 |
| **Continuous linear** | Intercept | -0.14 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.10 | 0.11 | 1.10 | 0.89 – 1.36 | 0.357 |
| Nasopharyngeal A1 HV4 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.13 | 0.12 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.58 | 0.50 | 1.79 | 0.68 – 4.75 | 0.241 |

# Table A3.6: Results of univariable ordinary logistic regression (OLR) analyses of the risk of ocular discharge with the 5 most prevalent *S. zooepidemicus* types

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| Tracheal wash A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.29 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.33 | 0.36 | 1.39 | 0.69 – 2.81 | 0.360 |
| **Ordered categorical** | Intercept | -1.32 | 0.16 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | 0.72 | 0.53 | 2.06 | 0.73 – 5.82 | 0.175 |
| | $\geq 10^3$ cfu/ml | 0.14 | 0.46 | 1.15 | 0.47 – 2.82 | 0.765 |
| **Continuous linear** | Intercept | -1.28 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.03 | 0.10 | 1.03 | 0.86 – 1.24 | 0.741 |
| Nasopharyngeal A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.20 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.24 | 0.40 | 0.79 | 0.36 – 1.73 | 0.553 |
| Tracheal wash A1 HVu *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.26 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.16 | 0.39 | 1.17 | 0.54 – 2.54 | 0.687 |
| **Ordered categorical** | Intercept | -1.30 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | 0.34 | 0.55 | 1.41 | 0.48 – 4.11 | 0.534 |
| | $\geq 10^3$ cfu/ml | 0.13 | 0.53 | 1.14 | 0.40 – 3.26 | 0.804 |
| **Continuous linear** | Intercept | -1.30 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.08 | 0.09 | 1.08 | 0.90 – 1.29 | 0.419 |
| Nasopharyngeal A1 HVu *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.23 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.03 | 0.40 | 0.97 | 0.44 – 2.13 | 0.931 |
| Tracheal wash A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.21 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.32 | 0.51 | 0.73 | 0.27 – 1.99 | 0.537 |
| **Ordered categorical** | Intercept | -1.24 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml* | - | - | - | - | - |
| | $\geq 10^3$ cfu/ml | 0.21 | 0.54 | 1.23 | 0.43 – 3.56 | 0.700 |
| **Continuous linear** | Intercept | -1.25 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.03 | 0.12 | 0.97 | 0.76 – 1.23 | 0.786 |
| Nasopharyngeal A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.25 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.15 | 0.54 | 1.16 | 0.41 – 3.32 | 0.780 |
| Tracheal wash C1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.20 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.51 | 0.56 | 0.60 | 0.20 – 1.81 | 0.367 |
| **Ordered categorical** | Intercept | -1.23 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml* | - | - | - | - | - |
| | $\geq 10^3$ cfu/ml | -0.10 | 0.58 | 0.91 | 0.29 – 2.84 | 0.870 |
| **Continuous linear** | Intercept | -1.25 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.04 | 0.12 | 0.96 | 0.77 – 1.21 | 0.753 |
| Nasopharyngeal C1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.22 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.21 | 0.48 | 0.81 | 0.32 – 2.07 | 0.661 |

Table A3.6 continued

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| Tracheal wash A1 HV4 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.23 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.02 | 0.58 | 0.98 | 0.31 – 3.09 | 0.975 |
| **Ordered categorical** | Intercept | -1.26 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml* | - | - | - | - | - |
| | $\geq 10^3$ cfu/ml | 0.35 | 0.61 | 1.42 | 0.43 – 4.67 | 0.568 |
| **Continuous linear** | Intercept | -1.29 | 0.15 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.10 | 0.11 | 1.10 | 0.88 – 1.37 | 0.389 |
| Nasopharyngeal A1 HV4 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.30 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.85 | 0.50 | 2.33 | 0.87 – 6.26 | 0.093 |

* No ponies with $<10^3$ $\log_{10}$ cfu/ml of this *S. zooepidemicus* type in tracheal washes had ocular discharge. Category dropped from the analysis to permit model convergence.

# Table A3.7: Results of univariable ordinary logistic regression (OLR) analyses of the risk of coughing with the 5 most prevalent *S. zooepidemicus* types

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| Tracheal wash A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.62 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.50 | 0.50 | 0.60 | 0.23 – 1.62 | 0.315 |
| **Ordered categorical** | Intercept | -1.59 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -0.42 | 0.77 | 0.65 | 0.14 – 2.97 | 0.582 |
| | $\geq10^3$.cfu/ml | -0.61 | 0.63 | 0.54 | 0.16 – 1.88 | 0.337 |
| **Continuous linear** | Intercept | -1.62 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.10 | 0.13 | 0.91 | 0.71 – 1.17 | 0.451 |
| Nasopharyngeal A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.83 | 0.18 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.39 | 0.41 | 1.47 | 0.66 – 3.30 | 0.347 |
| **Tracheal wash A1 HVu *S. zooepidemicus*** | | | | | | |
| **Binary** | **Intercept** | **-1.90** | **0.18** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Isolated** | **1.17** | **0.38** | **3.23** | **1.52 – 6.86** | **0.002** |
| **Ordered categorical** | **Intercept** | **-1.88** | **0.18** | | | |
| | **Not isolated** | **referent** | | | | |
| | **$<10^3$ cfu/ml** | **-0.20** | **0.77** | **0.82** | **0.18 – 3.71** | **0.793** |
| | **$\geq10^3$ cfu/ml** | **1.97** | **0.47** | **7.18** | **2.84 – 18.2** | **<0.001** |
| **Continuous linear** | **Intercept** | **-1.93** | **0.18** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Log cfu/ml$^{-1}$** | **0.37** | **0.09** | **1.45** | **1.22 – 1.74** | **<0.001** |
| Nasopharyngeal A1 HVu *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.76 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.002 | 0.47 | 1.00 | 0.39 – 2.53 | 0.996 |
| Tracheal wash A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.74 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.44 | 0.49 | 1.55 | 0.59 – 4.06 | 0.372 |
| **Ordered categorical** | Intercept | -1.71 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -0.37 | 1.07 | 0.69 | 0.08 – 5.66 | 0.729 |
| | $\geq10^3$ cfu/ml | 0.68 | 0.55 | 1.97 | 0.67 – 5.76 | 0.216 |
| **Continuous linear** | Intercept | -1.75 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.19 | 0.11 | 1.21 | 0.98 – 1.49 | 0.069 |
| Nasopharyngeal A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.74 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.46 | 0.76 | 0.63 | 0.14 – 2.82 | 0.546 |
| Tracheal wash C1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.69 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.01 | 0.57 | 0.99 | 0.32 – 3.00 | 0.979 |
| **Ordered categorical** | Intercept | -1.66 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -0.13 | 1.09 | 0.88 | 0.10 – 7.46 | 0.904 |
| | $\geq10^3$ cfu/ml | -0.01 | 0.65 | 0.99 | 0.28 – 3.53 | 0.982 |
| **Continuous linear** | Intercept | -1.67 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.03 | 0.12 | 1.03 | 0.81 – 1.31 | 0.809 |
| Nasopharyngeal C1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.75 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.16 | 0.56 | 0.85 | 0.28 – 2.55 | 0.771 |

Table A3.7 continued

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| **Tracheal wash A1 HV4 *S. zooepidemicus*** | | | | | | |
| **Binary** | Intercept | -1.72 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.47 | 0.59 | 1.60 | 0.50 – 5.10 | 0.425 |
| **Ordered categorical** | Intercept | -1.69 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | 0.60 | 1.17 | 1.81 | 0.18 – 17.8 | 0.610 |
| | $\geq 10^3$ cfu/ml | 0.39 | 0.67 | 1.48 | 0.40 – 5.54 | 0.557 |
| **Continuous linear** | Intercept | -1.71 | 0.17 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.14 | 0.12 | 1.15 | 0.91 – 1.44 | 0.250 |
| **Nasopharyngeal A1 HV4 *S. zooepidemicus*** | | | | | | |
| **Binary** | **Intercept** | **-1.89** | **0.17** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Isolated** | **1.44** | **0.51** | **4.20** | **1.54 – 11.5** | **0.005** |

## Table A3.8: Results of univariable ordinary logistic regression (OLR) analyses of the risk of abnormal breathing/dyspnoea with the 5 most prevalent *S. zooepidemicus* types

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| Tracheal wash A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.84 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.47 | 0.38 | 0.62 | 0.30 – 1.32 | 0.218 |
| **Ordered categorical** | Intercept | -0.80 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -1.22 | 0.77 | 0.30 | 0.07 – 1.33 | 0.112 |
| | $\geq10^3$ cfu/ml | -0.21 | 0.44 | 0.81 | 0.34 – 1.89 | 0.623 |
| **Continuous linear** | Intercept | -0.85 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | -0.03 | 0.09 | 0.97 | 0.81 – 1.16 | 0.726 |
| Nasopharyngeal A1 HV1 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.98 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.02 | 0.35 | 1.02 | 0.51 – 2.04 | 0.960 |
| **Tracheal wash A1 HVu *S. zooepidemicus*** | | | | | | |
| **Binary** | **Intercept** | **-1.01** | **0.14** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Isolated** | **0.71** | **0.35** | **2.03** | **1.02 – 4.02** | **0.043** |
| **Ordered categorical** | **Intercept** | **-0.98** | **0.14** | | | |
| | **Not isolated** | **referent** | | | | |
| | **$<10^3$ cfu/ml** | **0.53** | **0.50** | **1.69** | **0.63 – 4.54** | **0.297** |
| | **$\geq10^3$ cfu/ml** | **0.88** | **0.46** | **2.42** | **0.98 – 5.94** | **0.055** |
| **Continuous linear** | **Intercept** | **-0.97** | **0.14** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Log cfu/ml$^{-1}$** | **0.18** | **0.09** | **1.20** | **1.02 – 1.42** | **0.031** |
| Nasopharyngeal A1 HVu *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.99 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.11 | 0.37 | 1.11 | 0.54 – 2.30 | 0.769 |
| Tracheal wash A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.90 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.01 | 0.44 | 0.99 | 0.42 – 2.33 | 0.977 |
| **Ordered categorical** | Intercept | -0.87 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -0.39 | 0.81 | 0.68 | 0.14 – 3.35 | 0.635 |
| | $\geq10^3$ cfu/ml | 0.09 | 0.51 | 1.10 | 0.40 – 2.99 | 0.854 |
| **Continuous linear** | Intercept | -0.90 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.08 | 0.10 | 1.08 | 0.89 – 1.31 | 0.442 |
| Nasopharyngeal A1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.95 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Isolated | -0.44 | 0.57 | 0.65 | 0.21 – 1.99 | 0.448 |
| Tracheal wash C1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -0.95 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.48 | 0.42 | 1.62 | 0.70 – 3.71 | 0.259 |
| **Ordered categorical** | Intercept | -0.91 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | $<10^3$ cfu/ml | -0.002 | 0.85 | | | |
| | $\geq10^3$ cfu/ml | 0.60 | 0.48 | 1.00 | 0.19 – 5.25 | 0.998 |
| **Continuous linear** | Intercept | -0.91 | 0.13 | 1.81 | 0.70 – 4.68 | 0.219 |
| | Not isolated | referent | | | | |
| | Log cfu/ml$^{-1}$ | 0.11 | 0.09 | 1.11 | 0.92 – 1.34 | 0.269 |
| Nasopharyngeal C1 HV3 *S. zooepidemicus* | | | | | | |
| **Binary** | Intercept | -1.00 | 0.14 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.26 | 0.41 | 1.30 | 0.58 – 2.89 | 0.520 |

Table A3.8 continued

| Explanatory variable type | | β | S.E. β | OR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| **Tracheal wash A1 HV4 *S. zooepidemicus*** | | | | | | |
| **Binary** | Intercept | -0.95 | 0.13 | | | |
| | Not isolated | referent | | | | |
| | Isolated | 0.73 | 0.49 | 2.08 | 0.79 – 5.45 | 0.138 |
| **Ordered categorical** | **Intercept** | **-0.92** | **0.13** | | | |
| | **Not isolated** | **referent** | | | | |
| | **$<10^3$ cfu/ml** | **-0.18** | **1.16** | **0.84** | **0.09 – 8.15** | **0.877** |
| | **$\geq 10^3$ cfu/ml** | **0.92** | **0.55** | **2.51** | **0.85 – 7.38** | **0.095** |
| **Continuous linear** | **Intercept** | **-0.94** | **0.13** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Log cfu/ml$^{-1}$** | **0.23** | **0.11** | **1.26** | **1.02 – 1.56** | **0.034** |
| **Nasopharyngeal A1 HV4 *S. zooepidemicus*** | | | | | | |
| **Binary** | **Intercept** | **-1.05** | **0.13** | | | |
| | **Not isolated** | **referent** | | | | |
| | **Isolated** | **1.05** | **0.49** | **2.85** | **1.09 – 7.46** | **0.032** |